ANNUAL REPORTS IN MEDICINAL CHEMISTRY Volume 11

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

Editor-in-Chief: **FRANK H. CLARKE**

CIBA-GEIGY CORPORATION
ARDSLEY, NEW YORK

ACADEMIC PRESS New York and London 1976
A Subsidiary of Harcourt Brace Jovanovich, Publishers

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Volume 11

CONTRIBUTORS

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ACADEMIC PRESS New York San Francisco London 1976

A Subsidiary of Harcourt Brace Jovanovich, Publishers

Academic Press Rapid Manuscript Reproduction

ACADEMIC PRESS, INC.
111 Fifth Avenue, New York, New York 10003

United Kingdom Edition published by
ACADEMIC PRESS, INC. (LONDON) LTD.
24/28 Oval Road, London NW1

LIBRARY OF CONGRESS CATALOG CARD NUMBER: 66-26843

ISBN 0-12-040511-3

PRINTED IN THE UNITED STATES OF AMERICA

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Section Editor: T.Y. Shen, Merck & Company, Rahway, New Jersey

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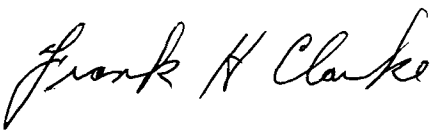
Section Editor: R.E. Counsell, University of Michigan, Ann Arbor, Michigan

PREFACE

Annual Reports provides timely and discriminating surveys of particular areas of medicinal chemistry. It is our policy not only to discuss new aspects of drugs that are in current use and the chemistry and biological properties of potential new drugs, but also to provide appraisals of new approaches to their discovery. Thus, Annual Reports is a convenient reference for locating the structure and properties of drugs in development. It also reviews those features of drug metabolism and concepts in structure-activity relationships, in the mechanisms of drug action and in receptor theory that are likely to influence drug discovery in the future.

This year the characterization of the opiate receptor and the isolation of endogenous analgetic peptides deserved special attention. The rapid advances in understanding membranes is also noted in this volume. Traditional areas have not been overlooked. Indeed, there are chapters on steroids, diabetes and anorectic agents. New concepts are illustrated with diagrams whenever possible.

The Editors of Annual Reports in Medicinal Chemistry welcome comments from its readers regarding the frequency of reviews on topics covered in past issues and suggestions for new topics which will make future volumes more useful.



Ardsley, New York
June, 1976

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Section I - CNS Agents

Editor: Maxwell Gordon, Bristol Laboratories, Syracuse, New York

Section Editorial

Progress has been made in a number of theoretical and applied areas and it can be expected that therapeutic advances may result from some of this research. However many problems still exist and this editorial will attempt to highlight both problems and progress.

In the area of the antipsychotics clozapine continues to appear to represent an improvement over earlier agents in respect to its relative lack of side effects. Continuing progress has been seen in the area of the development of antipsychotic agents with a long duration of action. In the area of antidepressants, there is still a great need for agents that act more rapidly in psychotic depressions, since a slow onset of activity carries with it a risk of suicide before the drug takes effect.

In the anticonvulsant area development has been completed, or nearly completed, on two new agents, clonazepin and eterobarb. The paucity of new anti-epileptic drugs has led the National Institute for Neurological Diseases and Stroke to sponsor work on the development of new agents, and as a result more progress can be expected in the future.

Among the analgetics butorphanol seems to offer promise as an addition to the physician's armamentarium. This drug is more potent than morphine, yet Lexington studies have shown it not to produce dependence of the opiate type. Research on the "opiate receptor" has yielded some fascinating results, especially in the finding of endogenous peptides with opiate-like activity, antagonized by naloxone. The finding of endogenous analgetic substances will undoubtedly stimulate the enunciation of many new concepts about the mechanism of pain and its relief by analgetics.

The medical management of the geriatric patient continues to be a major problem, and a growing one as life span is lengthened, the birth rate drops, and hence the proportion of the elderly in the population increases rapidly. The greatest need is to find agents that improve the cognitive function in the elderly so that they may remain functional longer and so that their requirement for institutional care may be delayed or reduced. Certain compounds in research, supplementing a very few already available commercially, offer the promise of improving the self-care of the elderly and alleviating such symptoms as confusion, unsociability, depression, etc. Other desirable CNS agents for the elderly would be hypnotics without the liability of producing ataxia or muscle weakness, appetite stimulants, improved antiparkinson agents with fewer side effects, etc.

Progress continues in the difficult area of the study of neurotransmitter substances and biogenic amines, which may lead to a better biochemical definition of mental illness. Such better understanding of biological factors in the psychosis and neurosis could hopefully lead to the design of more rational, target-specific drugs. CNS drugs developed by the screening approach, while very useful, almost invariably have a complex of biological

effects in addition to the desired one, so that side effects are almost inevitable.

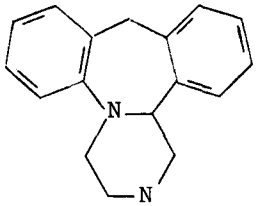
In the hypnotic area, further studies of the nature of sleep could lead to the development of newer agents which more nearly mimic natural sleep than do any of the existing drugs. The finding of natural "sleep factors" in the brain some years ago has not yet led to therapeutic progress, and this represents a research opportunity.

Chapter 1. Antidepressant and Antipsychotic Agents

P. F. VonVoigtlander, The Upjohn Company, Kalamazoo, Mich.

Introduction - This brief review highlights the progress in the last three years in the area of tricyclic antidepressants and the progress in the last year in antipsychotic agents. Included are compounds for which clinical efficacy has been recently established as well as those for which only early clinical and animal data are available. In addition, some of the newer concepts concerning the mechanisms of action of antidepressant and antipsychotic agents are discussed. For background information, the reader is directed to earlier volumes of this series[1,2]. For information of broader scope, several recent reviews are recommended[3,4,5].

Antidepressant Agents - In a double-blind comparison, mianserin (1, GB-94) has demonstrated antidepressant efficacy similar to amitriptyline[6]. Additional animal pharmacological studies[7,8] have revealed that this tetracyclic analog is similar to tricyclic antidepressants in several tests of antidepressant-like activity (potentiation of NE, and blockade of NE and 5-HT uptake). This is in contrast to earlier claims that mianserin "showed none of the pharmacological and biochemical prerequisites considered necessary for the prediction of antidepressant compounds"[9].



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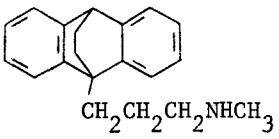

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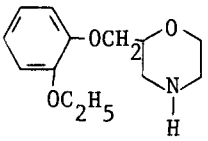

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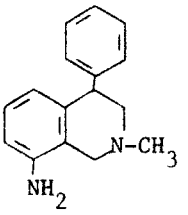
Another tetracyclic compound, maprotiline (2, Ludiomil®) has also displayed antidepressant activity in a double-blind clinical trial[10]. This compound is a potentiator of NE but has essentially no effect on 5-HT systems[11]. In a large multi-clinic trial, viloxazine (3, ICI 58834, Vivalan®) was clearly an efficacious antidepressant[12]. However, these studies did not substantiate earlier claims[13] that viloxazine had a faster onset of action than imipramine. Pharmacological studies indicate that viloxazine antagonizes reserpine, potentiates NE and blocks NE and 5-HT uptake[8,14].

In an open-label study of nomifensin (4), patients' hostility as well as depression appeared to be improved[15]. In animal studies, nomifensin blocked 5-HT and NE uptake, potentiated NE and antagonized reserpine[8,16,17]. Oxafluzane (5, 1766 CERM) displayed some antidepressant activity in open-label studies[18,19]. In addition it antagonized reserpine and oxotremorine and had antiaggressive effects in animals[20].



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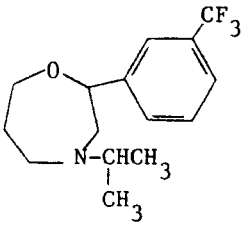

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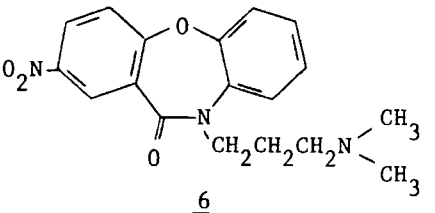
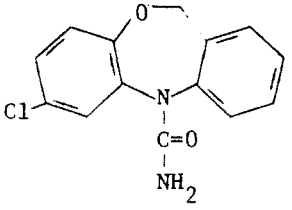


6

Sintamil (6) has been shown to be an effective antidepressant[21]. The pharmacological profile of this compound is similar to that of imipramine[22]. SQ-10996 (7) was well tolerated in early clinical testing[23]. Although it was originally evaluated as an anticonvulsant, animal studies suggested that SQ-10996 might have antidepressant-like properties[24].



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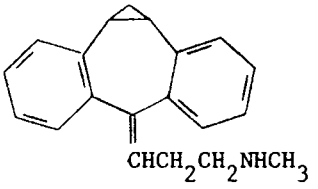

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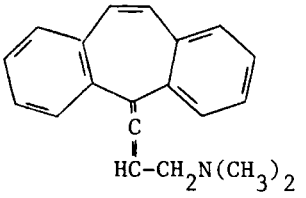
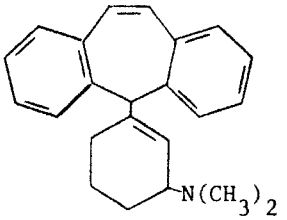


9

Over the past few years, a number of amitriptyline analogs have been synthesized and tested for antidepressant-like activity in animals. Introduction of increased rigidity in the central cycloheptyl ring (8) does not diminish anti-reserpine activity[25]. In addition, decreasing the flexibility of the side chain (9) enhances anti-reserpine activity. Compound 9 has also been shown to potentiate NE and to be more sedative than amitriptyline[26].



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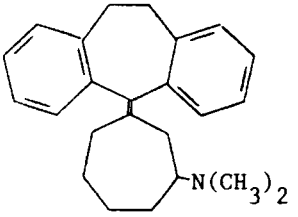

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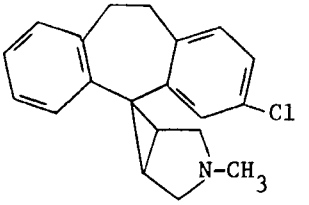
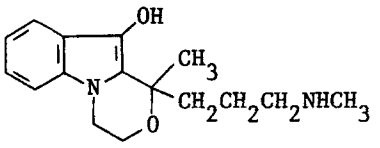


12

Amitriptyline related compounds in which the nitrogen is more or less rigidly fixed by cyclic substitution of the side chain display activity equivalent (10) to or less (11,12) than imipramine in the L-dopa potentiation test[27].

A number of novel tricyclic structures have also demonstrated antidepressant-like activities. An oxazino indole (13, AY-23673) possesses potent (12 times imipramine) anti-reserpine activity[28]. A series of pyrrolo-isoquinolines (14,15,16) displays potency greater than amitriptyline in a reserpine antagonism test[29].



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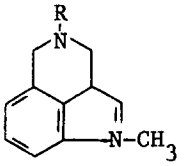
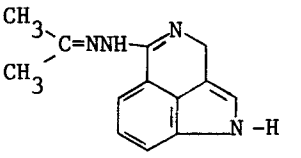
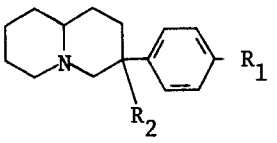


14 R = H
15 R = CH_2CH_3



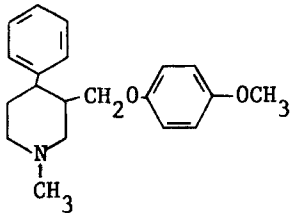
16

Arylquinolizidines containing a phenethylamine structure are more potent (17) than and equipotent (18,19) to imipramine in the L-dopa potentiation test[30,31].



17 $R_1 = OCH_3$, $R_2 = OH$
18 $R_1 = OCH_3$, $R_2 = H$
19 $R_1 = H$, $R_2 = H$

FG-4936 (20) is a potent blocker of 5-HT uptake *in vitro* but relatively inactive at blocking NE uptake[32]. In addition it potentiates 5-HT *in vivo*[33]. Similarly Lilly 110140 (21) is more potent than imipramine as a blocker of 5-HT uptake *in vitro* and *in vivo* but does not block NE uptake[34,35]. Lilly 94939 (22), on the other hand, potentiates NE but has minimal effects on 5-HT uptake[36].



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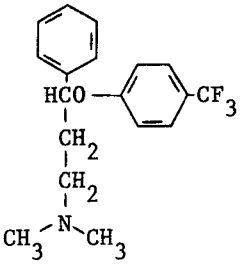

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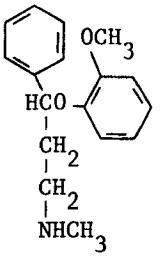
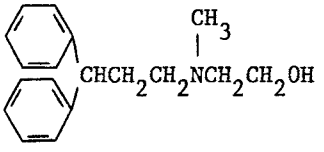


22

PF-82 (23) antagonizes reserpine with a potency greater than amitriptyline. In addition, it potentiates NE and blocks NE uptake[37]. An adamantanealkanamine (24), though a weak reserpine antagonist, potentiates NE responses[38]. The oxazolidone 69276-MD (25) is a weak antagonist of reserpine and a weak potentiator of amphetamine[39].



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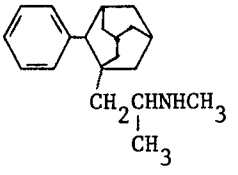

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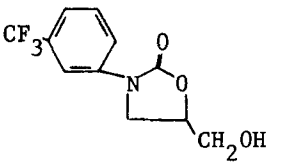

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In the preceding discussion, animal test systems involving antagonism of biogenic amine depletors and blockade of NE and 5-HT uptake have been used as predictors of antidepressant activity. This is not to imply that these tests are necessarily related to the clinical mechanism of action of antidepressants. Although most, but not all, clinically used antidepressants block either NE or 5-HT uptake[8] or dopamine uptake[40], there are apparently other mechanisms by which they potentiate biogenic amines. For example, in peripheral test systems, the ability of antidepressant drugs to potentiate noradrenergic responses does not correlate well with their ability to block NE uptake[41,42]. Clearly even the potentiation of NE responses cannot be relied upon exclusively to detect antidepressants because compounds that potentiate 5-HT preferentially[43,44] and 5-HT precursors[45] also display clinical antidepressant activity. It is possible, however, that one subclass of depressive illness is characterized by a deficiency in 5-HT function and another by a deficiency in NE function[46]. A recent study[47] of 5-HT and NE metabolite levels in the cerebrospinal fluid of depressed patients supports this idea. Perhaps selective blockers of NE[11,36] and 5-HT[32,35] uptake will prove particularly efficacious in the subclass of depressives deficient in the respective amine. Thus, although it is not known with certainty that the blockade of biogenic amine uptake is the mechanism of therapeutic action of antidepressants, drug development based on this hypothesis has yielded important pharmacological tools for the further study of depressive illness.

Another mechanism by which antidepressants may potentiate NE function is by an α-receptor mediated enhancement of NE release. Antidepressants are known to possess central α-receptor blocking effects as manifested by their ability to block the centrally mediated hypotensive effects of the α-agonist, clonidine[48]. Specific α-blockers have been shown to enhance NE turnover and to display unique stimulant effects[49]. In addition, several antidepressants have been shown to enhance NE release by a presumed pre-synaptic α-receptor blocking effect[50]. The importance of this mechanism to the clinical efficacy of antidepressants awaits the development and clinical testing of more selective pre-synaptic α-blocking agents.

The ability of antidepressants to block acetylcholine receptors is well known. Generally this property is considered undesirable because it results in peripheral anticholinergic side effects. Recently, however, interest in the central anticholinergic properties of antidepressants has been renewed. Janowsky *et al.*[51] have demonstrated that the cholinesterase inhibitor, physostigmine, exacerbates depression and alleviates mania. This implies that the anticholinergic properties of antidepressant agents may be important in the therapeutic effect of these compounds.

This discussion of antidepressants has been restricted to the tricyclic compounds or those which are not inhibitors of monoamine oxidase (MAO). Certain *in vitro* studies[52,53], however, have indicated that tricyclic antidepressants might also display significant MAO inhibitory activity. Specifically, they demonstrated an inhibition of MAO mediated phenethylamine (PEA) metabolism by several tricyclic antidepressants. This might be of particular interest because other workers have observed that the pre-

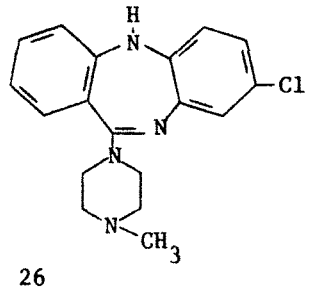
cursor of PEA, phenylalanine, appears efficacious in the treatment of depression[54]. However, tricyclic antidepressants in contrast to classical MAO inhibitors, do not alter the in vivo metabolism of PEA[55].

A recent study[56] has confirmed that depressives excrete abnormally low amounts of cyclic adenosine monophosphate (cAMP) and that these amounts increase when the patient is treated with antidepressants. Animal data[57] indicate that these drugs elevate cAMP concentrations in the brain presumably by inhibiting phosphodiesterase. These results are of particular interest because of the involvement of adenyl cyclase with the adrenergic receptor. However, the effects of tricyclic antidepressants upon NE-stimulated cAMP formation in vitro do not correlate with the effects of antidepressants on cAMP excretion or brain concentration; incubation with imipramine[58] or protriptyline[59] or chronic in vivo treatment with desmethylimipramine or iprindole[60] decreases the in vitro cAMP response to added NE. Perhaps this inhibition of NE-stimulated adenyl cyclase is related to the α-adrenergic receptor blocking properties of antidepressants.

A major problem in attempting to relate the mechanisms involved in the animal studies of antidepressants to the clinical mechanism of action of these compounds is the time course of the effects. In all of the aforementioned animal systems, the response to the antidepressant is not qualitatively different upon acute or chronic dosing. Clinically, however, antidepressant effects appear only after chronic dosing[61]. This, in fact, is still a major drawback of currently available antidepressants. An understanding of why antidepressant agents require chronic treatment and/or the development of animal test systems that predict the slow onset of action of classical antidepressants would be an important step toward developing rapid acting, more highly efficacious antidepressants.

Antipsychotic Agents - Clinical studies on clozapine (26), a dibenzodiazepine antipsychotic that causes few extrapyramidal symptoms (EPS)[62] have continued. In a double-blind cross-over experiment, patients receiving clozapine displayed no EPS, no tardive dyskinesia, and no elevations in cerebrospinal fluid homovanillic acid (HVA)[63]. This was in complete contrast to the effects of haloperidol in the same patients. Although some problems with agranulocytosis have occurred with this compound, the overall incidence is no higher than with other neuroleptics[64].

Additional clinical studies have been performed with pipothiazine palmitate (27), a long-acting phenothiazine antipsychotic[65]. This compound has demonstrated efficacy equivalent to fluphenazine enanthate in a double-blind study in chronic schizophrenics[66]. In addition, a single injection of pipothiazine palmitate had a duration of action of 4 weeks as compared to 2 weeks for fluphenazine enanthate. Su-23397 (28) appeared to be an efficacious antipsychotic agent in an open label study[67]. However, EPS were also noted. In animal studies, clopimizide (29, R-29764) displays a much longer duration of action than its non-halogenated analog pimozide[68].



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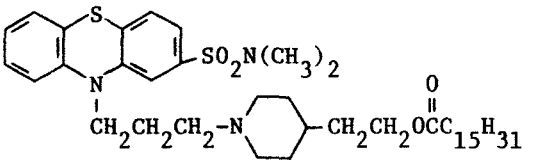

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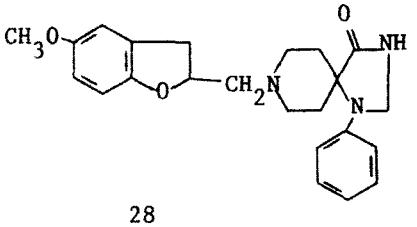

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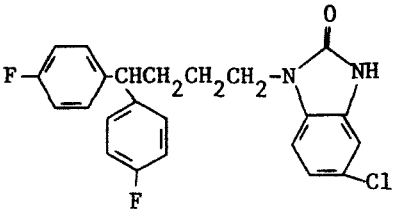

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The proposed relationship of dopaminergic systems to the mechanism of antipsychotic activity and EPS has been adequately reviewed in earlier volumes of this series[2,69]. Briefly, antipsychotic agents block dopamine receptors in the corpus striatum and ventral limbic areas. The blockade in the striatum is thought to result in EPS whereas blockade in the ventral limbic area (or limbic and frontal cortices[70]) results in the antipsychotic effects. Activation of the dopamine receptor results in an elevation in post-synaptic cAMP. Antipsychotic agents block dopamine-stimulated elevations in cAMP. In addition, blockade of dopamine receptors results in an enhancement of dopamine turnover through direct pre-synaptic and/or multi-synaptic feedback mechanisms. This activation of pre-synaptic tyrosine hydroxylase by antipsychotics is mediated by an increased affinity of the enzyme for its cofactor. Cholinergic systems appear to modulate the effects of striatal, but not limbic, dopamine inputs. Thus anticholinergic agents block EPS but not antipsychotic efficacy. Likewise drugs that combine dopamine blocking and anticholinergic properties are efficacious antipsychotics with low EPS potential.

Within this frame of concepts, several interesting discoveries have been made in the last year. Although dopamine-stimulated adenyl cyclase has been thought to be involved with the dopamine receptor, efforts to study the actual binding of dopamine agonists and antagonists to the dopamine receptor were not successful until recently. Two independent laboratories[71,72] have now reported that a significant portion of H^3-dopamine and H^3-haloperidol binding to brain tissue homogenates is stereospecific as defined by the ability of the active (+) but not the inactive (-) isomer of butaclamol[73] to block it. This stereospecific binding is blocked almost exclusively by dopamine agonists and antagonists (antipsychotic agents). Both groups report that binding IC50's for a wide range of antipsychotics correlate well with the clinical potency of the drugs. Thus, this binding activity may represent the dopamine receptor and these assays

may serve as in vitro screening procedures for antipsychotic-like activity.

Recent experiments concerning the sites of action of antipsychotics on dopamine-containing cells have produced some surprising results[74]. Injection of haloperidol into the striatum depressed, rather than enhanced, the firing of dopaminergic cells. Only when haloperidol was injected in the region of the dopaminergic cell bodies in the substantia nigra was activation of the nigral cells observed. Thus multi-synaptic feedback from the striatum to the substantia nigra may not mediate the enhancement of dopamine turnover induced by antipsychotics. The authors suggest instead that antipsychotics may block a self-inhibition process whereby the dendrites of dopamine cells release dopamine on to the cell soma.

As regards the molecular mechanisms of the feedback enhancement of dopamine turnover, workers in Costa's laboratory[75] have shown that antipsychotic drugs with low EPS potential, in contrast to those which cause EPS, increase the affinity of tyrosine hydroxylase for its cofactors to a much greater degree in the ventral limbic region than in the corpus striatum. This may be due to the anticholinergic activity of the low EPS antipsychotics[76]. Regardless, measuring the regional effects of antipsychotic drugs on the affinity of tyrosine hydroxylase for its cofactor offers another technique to predict low EPS liability.

Additional information has been provided on the interaction of antipsychotic drugs with cholinergic systems in the striatum. Apomorphine has been shown to enhance acetylcholine turnover in this region. All antipsychotic drugs tested (including clozapine) block this effect[77]. However, the decrease in striatal acetylcholine turnover induced by cholinomimetics is blocked by clozapine but not by other antipsychotic agents[78]. This is further evidence that anticholinergic activity distinguishes clozapine from other antipsychotics with greater EPS liability.

An extensive body of knowledge now suggests links between the actions of antipsychotic drugs and dopamine systems[79]; however, this does not preclude the possibility that interaction with other neurotransmitter specific systems might be therapeutic in treating the psychoses, particularly if these systems in turn selectively modulate particular dopaminergic pathways. Baclofen [β-(p-chlorophenyl)-γ-aminobutyric acid], a compound that has been found useful in the treatment of spasticity[80], has displayed some antipsychotic-like effects in animal behavioral test systems[81]. When administered to schizophrenics, baclofen appears to allow the antipsychotic dose to be lowered and in addition appears to result in a greater efficacy than that seen with the antipsychotic alone[82]. Although the mechanism of these effects is not known with certainty, the observation that γ-aminobutyric acid (GABA) neurons exert an inhibitory influence on dopamine cells may be of importance[83], particularly in light of the supposed GABA agonist properties of baclofen[84]. In support of these contentions, baclofen has been shown to antagonize the elevations in dopamine turnover induced by pimozide[84]. Thus investigations into the interactions between GABA and dopamine systems may provide the groundwork for developing a new class of antipsychotic agents.

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Chapter 2. Anti-Anxiety Agents, Anticonvulsants, and Sedative-Hypnotics

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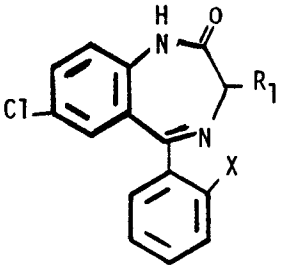
Introduction - Benzodiazepine derivatives and analogs continue to dominate the pharmacologically related areas of anti-anxiety, anticonvulsant, and sedative-hypnotic therapy. Novel structures such as some of those reported in this review may provide leads to compounds that show a greater specificity of central nervous system depressant activity.

Anti-Anxiety Agents - Benzodiazepines and Related Compounds - Patients given 15-30 mg/day diazepam over several months showed higher concentrations of N-desmethyldiazepam (1a) in plasma and cerebrospinal fluid than did patients given no diazepam prior to acute treatment.[1] This study was interpreted as indicating that a cumulation of this psychoactive metabolite occurs during long-term treatment with diazepam. Dasberg[2] found that 15 mg desmethyldiazepam daily was "a more effective hypnosedative and mood-lowering substance" than equal doses of diazepam.

The animal and clinical pharmacology of oxazepam (1b) was reviewed[3]. Lorazepam (1c) was reported to be effective as an anti-anxiety agent in doses of 1-6 mg/day.[4-7]

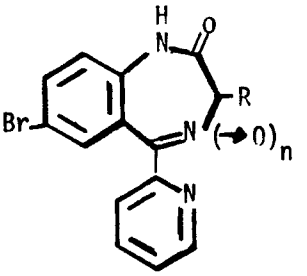
Bromazepam (2a) was reported to be effective as an anti-anxiety agent in doses of 15-30 mg daily.[8-10] Two metabolites of bromazepam in mice were found to be 2b and 2c. All 3 compounds showed good anti-pentylenetetrazol activity.[11]



1a, R_1 = X = H

1b, R_1 = OH, X = H

1c, R_1 = OH, X = Cl



2a, R = H, n = 0

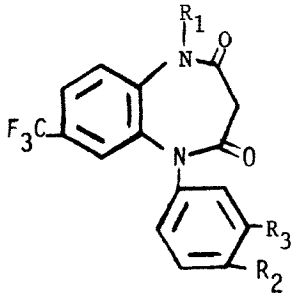
2b, R = OH, n = 0

2c, R = H, n = 1

Triflubazam (3a) was reported to be equipotent with diazepam in mice against electroconvulsive shock and was 1/2-1/7 as potent as diazepam in antagonizing shock-or isolation-induced aggressive behavior.[12] The predominant metabolites of triflubazam in man appear to be 3b-d.[13]

A 28-day double-blind assessment of diazepam 15 mg daily and chlorazepate dipotassium (4) 22.5 mg daily indicated that the two drugs had slightly different modes of action.[14] Diazepam depressed baseline and stimulation arousal whereas chlorazepate decreased baseline arousal but facilitated central nervous system response upon stimulation. These differences were not statistically significant on psychological rating scales, but measurement of several physiological parameters supported the psychometric data.

Pinazepam (5) was equipotent with diazepam in antagonizing footshock-induced aggressive behavior, pentylenetetrazol convulsions and electroshock convulsions in mice.[15] Another novel benzodiazepine, ID622 (6), was 3-10 times more potent than diazepam in antagonizing isolation-induced aggression in mice.[16]

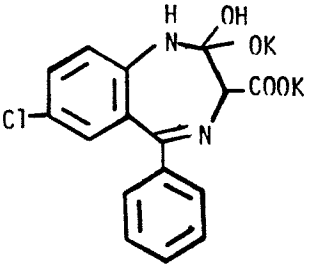


3a, R_1=CH_3, R_2=R_3=H

3b, R_1=R_3=H, R_2=OH

3c, R_1=H, R_2=OH, R_3=OCH_3

3d, R_1=H, R_2=R_3=OH



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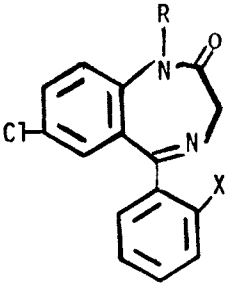
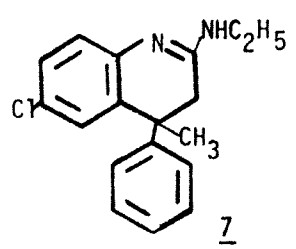


5, R = $-CH_2C{\equiv}CH$, X = H

6, R = $-CH_2CH_2-\overset{O}{\overset{\|}{S}}(=O)-CH_3$, X = F

Non-benzodiazepine Anti-Anxiety Agents - Etafenoxin (7) in an uncontrolled clinical trial produced improvement in patients with anxiety at doses of 100-260 mg/day.[17] Trazodone (8) in an uncontrolled clinical trial produced improvement in patients with anxiety at doses of 60-150 mg/day.[18] Administration of 30 mg benzoctamine (9) daily for 2 weeks under double-blind conditions to outpatients with anxiety neurosis significantly affected the symptom of anxiety when evaluated by a rating scale.[19]



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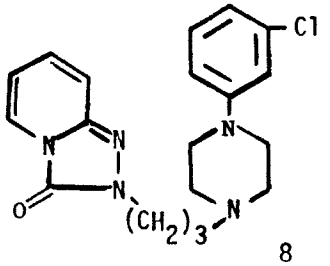

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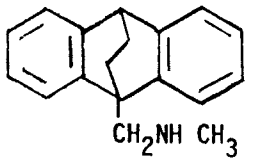

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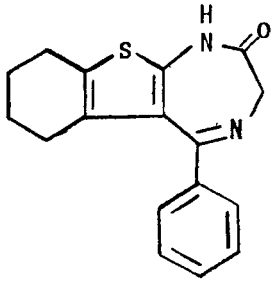
Bentazepam (thiadipone) (10) was 1/3 as potent as chlordiazepoxide in antagonizing isolation-induced aggression in mice and was 1/2 as potent in antagonizing electroshock convulsions.[20] A pilot study with SCH 12679 (11) indicated that it was effective in treating behavioral disorders at a daily dose of 300-400 mg.[21] Compound YG19-256 (12) blocked shock-induced aggression in rats at i.p. doses of 5-10 mg/kg.[22]



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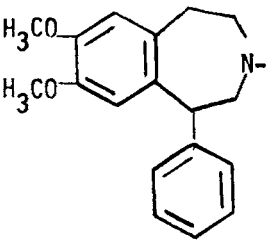

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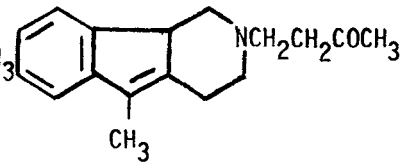

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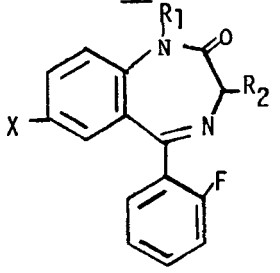
Sedative-Hypnotics - Benzodiazepines and Related Compounds - The pharmacology of flurazepam (13a) was reviewed.[23] Clinical studies[24,25] indicated that flurazepam was an effective hypnotic agent in doses of 15-30 mg. Kales[26] found that flurazepam was effective in inducing and maintaining sleep with long-term use (4 weeks) while pentobarbital was effective only with short-term administration. Compound SAS643 (13b) was approximately twice as active as flurazepam in causing sedation and inducing sleep in rats treated with a subhypnotic dose of hexobarbital.[27] In a clinical trial[27], 5 mg SAS643 was as effective as 15 mg flurazepam in inducing sleep. Clinical studies with flunitrazepam (13c) indicated that it was an effective hypnotic at doses of 1-3 mg.[28-30]

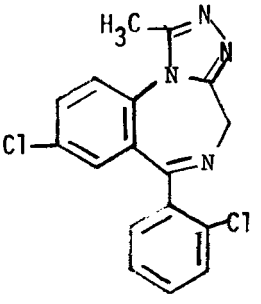
Triazolam (14) was an effective hypnotic in doses of 0.5-1 mg.[31-34] Ketazolam (15) had similar hypnotic activity.[35]



13a, $R_1=(CH_2)_2N(C_2H_5)_2$, R_2=H, X=Cl

13b, $R_1=CH_2CH_2OH$, R_2=OH, X=Cl

13c, $R_1=CH_3$, R_2=H, X=NO_2



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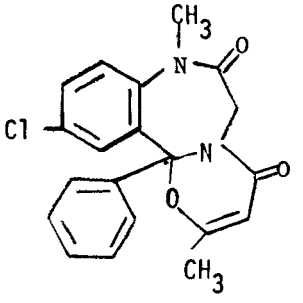
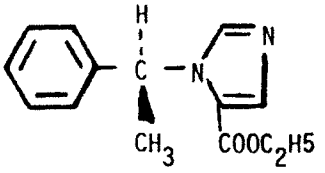


15

Non-benzodiazepine Sedative-Hypnotics - The clinical pharmacology of drugs used to improve sleep was reviewed.[36] Etomidate (16), a novel intravenous anesthetic agent, produced hypnosis in several laboratory animal species in doses of 0.14-1.1 mg/kg.[37] The (R)-(+)-enantiomer was found to show all the pharmacologic activity of the racemic mixture.[38] Promethazine (17) showed a dose-related depression of REM sleep but caused a weaker REM rebound after withdrawal than did pentobarbital.[39] This property of promethazine may explain its usefulness in the treatment of withdrawal symptoms following alcohol and barbiturate abuse.



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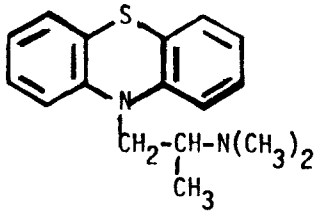
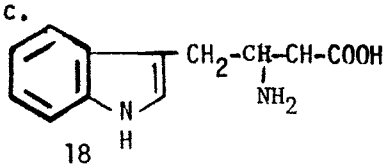


17

Tryptophan (18) given intravenously (100 mg/kg) or orally (5 g) produced an increase in drowsiness in healthy volunteers.[40-41] The first pharmacologic evidence was reported for a direct hypnogenic effect of piperidine in the central nervous system.[42] The results suggested a possible involvement of piperidine in the process of sleep. Aboul-Enein[43] reported on the hypnotic and anticonvulsant properties of gluthethimide (19a) metabolites and analogs. Compound 19b, a metabolite of gluthethimide, was similar in potency to the parent compound. Compound 19c had similar anticonvulsant potency but was approximately 1/3 as active as a hypnotic.



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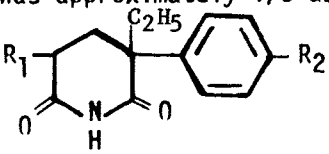


19a, $R_1=R_2=H$

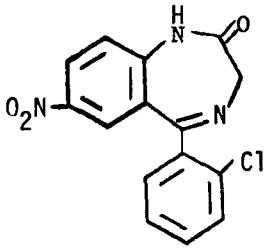
19b, $R_1=OH, R_2=H$

19c, $R_1=H, R_2=NH_2$

The relationships between lipophilicity (log partition coefficient) and iso-effective doses (log 1/isoeffective molar drug concentration) of aliphatic hydrocarbons, ethers, and ketones (C5-C17 chain length) were determined after i.v. injection in mice.[44] The dose causing loss of righting reflex was determined. Anesthetic activity reached a peak at a chain length of C8-C10 and then decreased for all 3 groups of compounds. This relationship could be expressed mathematically by the parabolic equation $\log 1/C = a (\log P)^2 + b (\log P) + c$.

Anticonvulsants - A primate model for testing anticonvulsant drugs was reported by Meldrum.[45] Senegalese baboons with a natural syndrome of photosensitive epilepsy showed generalized myoclonic jerks if stimulated stroboscopically at hourly intervals 2-8 hours after the intravenous administration of 200 mg/kg allylglycine. Drugs active against grand mal seizures (phenobarbital, diazepam, carbamazepine, diphenylhydantoin) were active on this test system while sulthiame and ethosuximide had little antiepileptic activity.

Clonazepam (20) was effective in controlling epileptic and other motor seizures in doses of 0.5-10 mg daily.[46-48] Eterobarb (21) was effective clinically in controlling seizures at daily doses of 2-8 mg/kg.[49] Sedation did not appear to be as prominent with this barbiturate as with phenobarbital. Aminomethsuximide (22) showed anti-pentylenetetrazol activity in mice similar to that of the parent compound.[50] The compound was inactive against electroshock convulsions.



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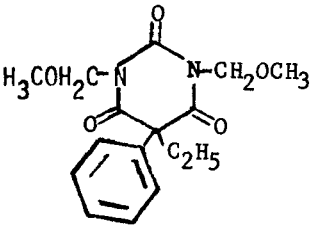

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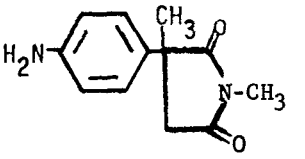
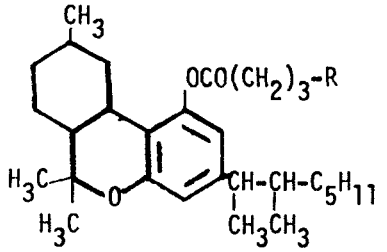
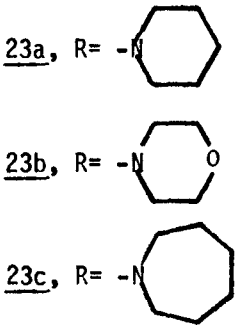


22

Three cannabinoids (23a-c) were more active than diphenylhydantoin against electroconvulsive shock in rats.[51] In a series of derivatives of diphenylhydantoin (DPH), the most active compound was 24 which had 1/3 the potency of DPH against electroconvulsive shock.[50]

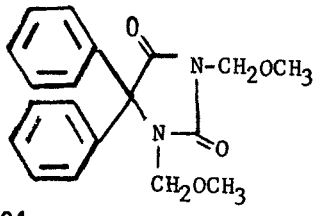




23a, R= -N

23b, R= -N O

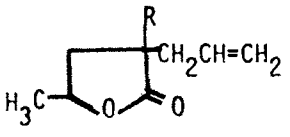
23c, R= -N



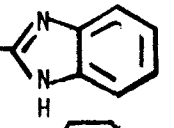
24

In a series of substituted γ-butyrolactones, compounds 25a and 25b were the most active against electroconvulsive shock.[53] A series of isomeric octahydrophenanthrenes were found to have anti-electroshock and anti-pentylenetetrazol activity.[54] Compounds 26a and 26b were approximately 1/2 as potent as meprobamate against electroshock.

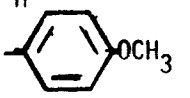
Cyclobenzaprine, a novel centrally acting muscle relaxant (27), was 1/2 as potent as diazepam in antagonizing electroshock convulsions and 1/4 as potent in antagonizing pentylenetetrazol convulsions.[55] In a series of N-substituted succinimides, compound 28 was very active against electroshock convulsions, with an ED_{50} of approximately 5 mg/kg.[56] Compound 29 was the most active antagonist of pentylenetetrazol in a series of cinnamamides.[57] It had an ED_{50} of 50 mg/kg.

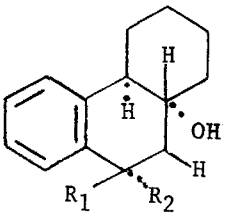


25a, R=



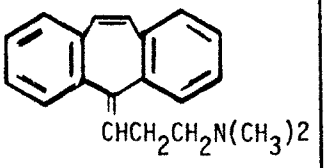
25b, R=-CONHCONH-

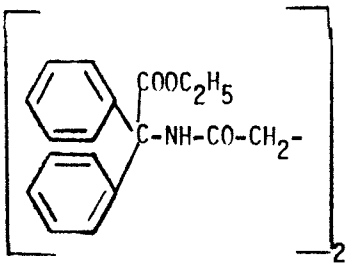


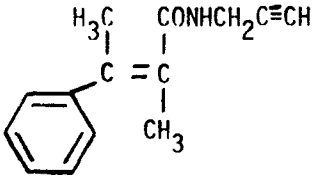


26a R_1= H, R_2= OH

26b R_1= OH, R_2= H

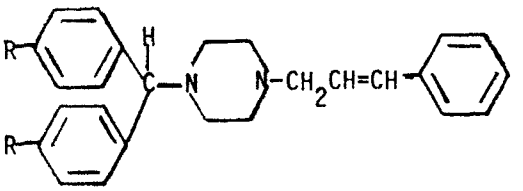






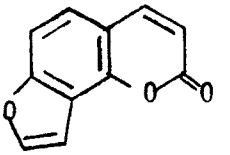
27 28 29

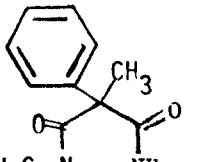
The anticonvulsant properties of cinnarizine (30a) and flunarizine (30b) were studied in mice and rats.[58] Flunarizine had 1/3 the potency of diphenylhydantoin in antagonizing electroconvulsive seizures while cinnarizine was 1/7 as potent. Angelicin (31), a compound isolated from Selinum vaginatum, was approximately 1/2 as potent as chlordiazepoxide in reducing spontaneous activity of mice and was approximately 1/10 as active as chlordiazepoxide in antagonizing electroshock.[59] A series of substituted pyrazolinediones showed anticonvulsant activity.[60] The most active compound was 32 which was approximately 1/2 as potent as phensuximide in antagonizing pentylenetetrazol convulsions.



30a, R=H

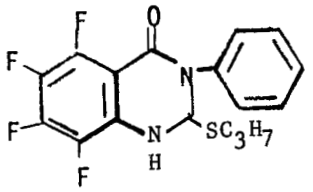
30b, R=F





31 32

Compounds 33-39 showed weak anticonvulsant activity and were the most active in their respective series.[61-67] The compounds, at a dose of 100 mg/kg i.p., gave 70-90% protection against pentylenetetrazole convulsions.



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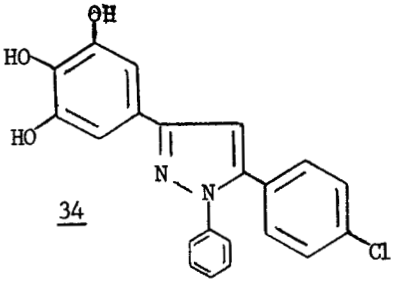

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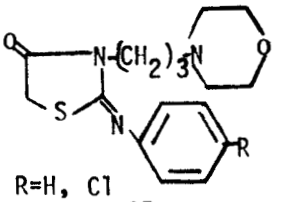
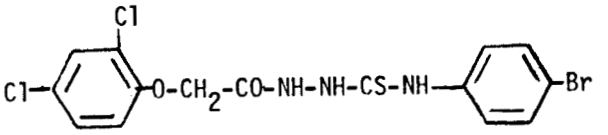


R=H, Cl

35



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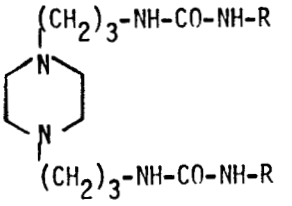
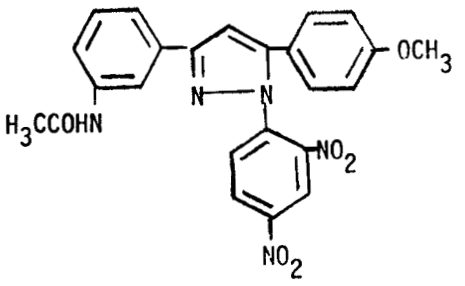


R = o-or p-methoxyphenyl

37



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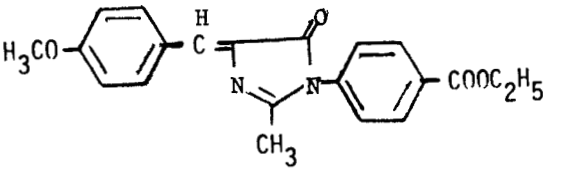

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Chapter 3. Narcotic Antagonists and Analgesics

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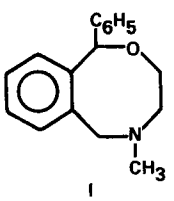
Introduction – The characterization of enkephalin, an endogenous morphine-like factor, and the opiate receptor represent major advances in the understanding of opiate mechanisms. These topics are reviewed here and in Chapter 4. The report of a benzomorphan series possessing potent analgesic activity without physical dependence capacity highlighted this year's SAR studies. Biospecificity remains the key issue for the potential development of cannabinoids as analgesics. A brief review has appeared covering the opiate receptor and the endogenous opiate.[1] The annual evaluation of advanced agents for physical dependence in monkeys was issued.[2]

Clinical Studies – Good parenteral analgesia has been reported in advanced clinical trials with the structurally unique analgesic nefopam (1). In double blind trials assessing intramuscular potency, nefopam (20 mg) was as effective as meperidine[3] (40 mg) and morphine[4] (12 mg) and was without significant respiratory depression.[5] In one trial orally administered nefopam (60 mg., t.i.d.) was shown to be distinctly superior to placebo in analgesic effectiveness.[3] In another study,[6] oral nefopam (2 x 30 mg) exhibited greater pain relief than aspirin (300 mg) on day 1; no significant difference from aspirin or placebo was seen on days 2 and 3. Intramuscular butorphanol (2a) was found to be approximately 20 times as potent as pentazocine in post-operative patients with drowsiness being the only side effect for either drug at the high doses (5 and 60 mg, respectively).[7] In another double-blind study, doses of 2 mg or greater of butorphanol caused psychotomimetic reactions.[8] The synthesis and SAR which led to the development of butorphanol and oxiloriphan (2b) was reported.[9,10,11] Animal pharmacology on WY-16,225 (3) characterized it as both a potent agonist (17 x morphine, mouse hot plate) and antagonist lacking addiction liability and possessing no serious cardiovascular or respiratory side effects.[12] However, in dose range finding studies in man no significant analgesic activity was evident at doses up to 4.8 mg, i.m.[13]



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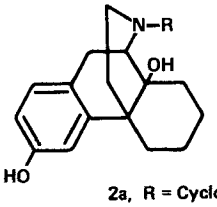
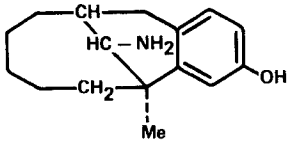


2a, R = Cyclobutylmethyl

2b, R = Cyclopropylmethyl



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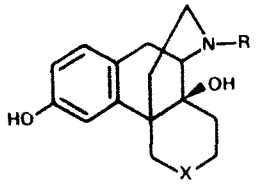
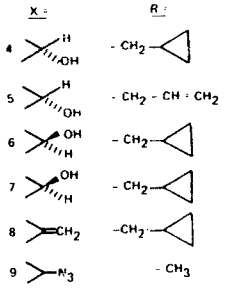
The anthranilate, floctafenine (RU 15750) exemplifies the increasing use of antiinflammatory/analgesic agents for the treatment of non-arthritic pain. In clinical trials with patients suffering from post-operative pain, floctafenine (200 mg) was as effective an analgesic as aspirin (600 mg),[14] pentazocine (50 mg),[15] dihydrocodeine (60 mg),[16] and d-propoxyphene (130 mg)[17] with fewer side effects. Floctafenine was also shown to be an effective analgesic in osteoarthritis.[18] The detailed animal pharmacology of floctafenine has been published.[19] Efficacy in post-operative pain was also reported for the antiinflammatory/analgesics indoprofen,[20] MK-647,[21] and fenbufen.[22,23]

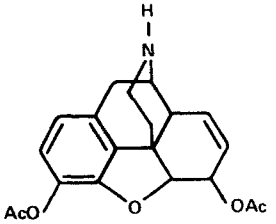
Structure Activity: Strong Analgesics

Morphines, Morphinans – The agonist-antagonist profiles of naltrexone and naloxone were shown to be highly sensitive to functional change at the 6-carbonyl position. Significant antinociceptive activity was demonstrated for 4 (≈ nalorphine) and 5 (≈ pentazocine) in the mouse writhing test.[24] While the

6α-metabolites (**4** and **5**) were more active than the 6β-derivatives (**6** and **7**) as analgesics, it was reported that neither epimeric series exhibits antagonist activity.[25] Derivative **8**, in which methylene replaces the ketone, was a potent, pure narcotic antagonist.[26]







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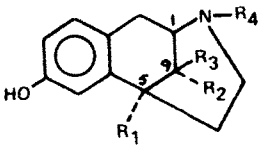
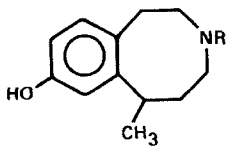
In additional work relevant to the SAR and clinical activity of the opiates, 14-hydroxyazidomorphine (**9**) was shown to possess analgesic activity and a relatively low dependence capacity comparable to that of its parent, azidomorphine.[27] The previously unreported nor-heroin (**10**) was found to be 20 times less potent than heroin (mouse hot plate) and to resemble morphine in its onset, peak and duration of analgesic action.[28] Drug distribution coefficients for a number of narcotic agonists and antagonists were found to be extremely sensitive to pH changes in the human physiological range.[29]

Benzomorphans – Several pharmacologically unique compounds emerged from this class during the past year. One of the most interesting reports covered a series of N-substituted tetrahydrofurfuryl benzomorphans.[30] The most active compound (**11**, absolute configuration, 1R/5R/9R/2"R) was 50 times more potent than morphine (mouse writhing), did not produce Straub tail in mice, had a high therapeutic ratio (LD_{50}/ED_{100} = 9,400), failed to suppress abstinence signs in withdrawn morphine dependent monkeys, and did not show antagonist properties. The diasteriomers, **11** and **12** with the 2" R configuration, had significantly greater analgesic activity than their corresponding 2" S isomers. It is of interest to note that **11** and ketocyclazocine share many of the unique pharmacological properties of the endogenous opiate, enkephalin (see below and also Chapter 4).



	R_1	R_2	R_3	R_4
11	CH_3	CH_3	H	$-CH_2-$ (2"R-furfuryl)
12	CH_3	H	CH_3	$-CH_2-$ (2"-furfuryl)
13	CH_3	H	CH_3	CH_3
14	CH_3	H	C_2H_5	CH_3
15	CH_3	H	C_3H_7	CH_3
16	H	H	CH_3	CH_3
17	CH_3	CH_3	OH	$CH_2-CH=C(CH_3)_2$
18	CH_3	CH_3	OH	$CH_2-c-C_3H_5$
19	CH_3	CH_3	H	$(CH_2)_3-C(=O)-C_6H_4-F$



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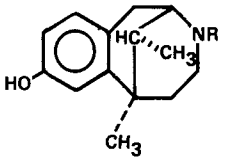
Substitution at the 9β position in the benzomorphans increased agonist or antagonist activity. For example, the 9β-alkyl derivatives, 13-15, were 3-5x more potent as analgesics than their 9α-alkyl epimers (mouse hot plate).[31] The 9β-methyl derivative, **16**, had analgesic activity in mice equal to morphine, did not support morphine dependence in monkeys and in fact precipitated withdrawal at 1 mg/kg.[32] It was further found that 9β-hydroxylation increases the antagonist activity (**17** 3-4 x pentazocine; **18** 100 x cyclazocine) and decreases the agonist activity (cyclazocine, 400 x **18**) in the benzomorphan series.[33]

The novel benzomorphan, **19** (ID-1229), was advanced as a potent, non-narcotic analgesic (3-5 x pentazocine in the mouse writhing test) with a significant degree of CNS depressant activity.[34] The benzazocine homolog **20** (R=C_6H_{13}) was ten fold less active than its rigid counterpart **21** (mouse hot plate).[35] *In vitro* opiate receptor binding strength correlates well with *in vivo* analgesic activity in the homologous series **20** (R=CH_3 thru C_7H_{15}) while no such correlation exists for the benzomorphan series (**21**, R=CH_3 thru C_6H_{13}). The simple benzomorphan C ring homolog **22** was nearly as active as codeine whereas the hydroxylated derivative **23** was forty times less potent than morphine.[36] The newly synthesized pentazocine analog, **24**, was found to possess one-fourth the agonist activity of pentazocine (mouse writhing).[37]



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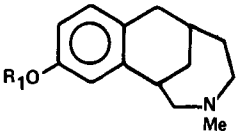
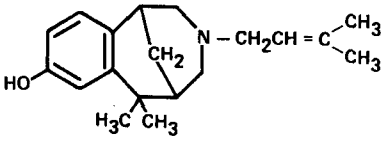


22 R_1 = Me
23 R_1 = H



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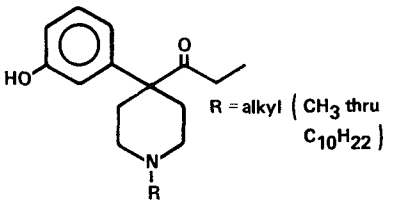
Prodines and Related Structures – The N-pentyl norketobemidone derivative **25** (R=C_5H_{11}) was found to be the most potent analgesic in a series of ten homologs (mouse hot-plate, ED_{50} = 0.78).[38] Activity in this series was statistically correlated with relative opiate receptor binding affinity and agonist activity in the guinea-pig ileum.[38,39] In another study, the analgesic activity of meperidine and three N-alkyl homologs was found to be proportional to their relative brain levels.[40]



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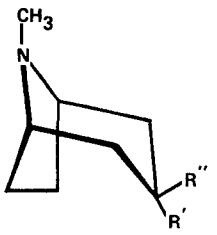
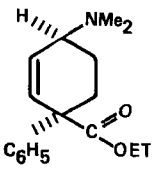


26 R' = CO_2Et, R'' = C_6H_5
27 R' = C_6H_5, R'' = CO_2Et



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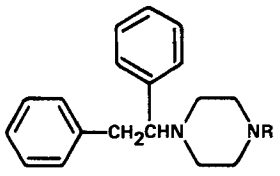
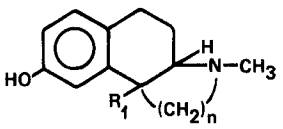
A new tropane analog of meperidine, **26**, was reported to be 3-5x less potent than its epimer **27** and meperidine (tail flick).[41] The positional isomer **28** of tilidine, was found to be more potent than the parent (6x tilidine, s.c., mouse phenylbenzoquinone writhing) and to possess neuroleptic properties.[42]

Miscellaneous – In a series of N-substituted piperazines, the cycloalkyl derivatives (29-31) were reported to have morphine equivalent analgesic activity (mouse hot plate and writhing, rat tail clamp).[43] The cis and trans isomers (32 R_1=C_2H_5, n=3) from a series of octahydrobenzo[f] quinolines and benz[e] indoles were as potent as morphine (mouse hot plate).[44] The most potent derivative (33) in a series of reduced benzazepines and benzazocines[45-49] had analgesic activity 1.4 x morphine in the mouse writhing test. Atropine antagonized the analgesic activity of 33, suggesting a cholinergic component in the analgesic activity of this class.



29, R = c – C_6H_{11}
30, R = c – C_7H_{13}
31, R = c – C_8H_{15}



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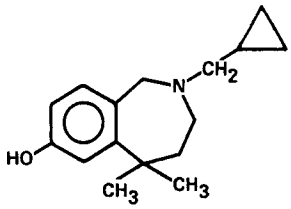
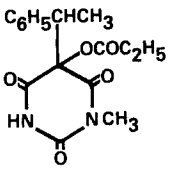


33

In continuing studies on the 5-phenethyl barbituate analgesics, the best activity in an N-alkylated series was seen for 34 (10 x codeine, p.o., mouse hot plate).[50] The synthesis of 35, a new metabolite of acetylmethadol, was reported.[51] This compound was as potent as morphine (mouse writhing)[52] suggesting that it may contribute to the analgesic activity of acetylmethadol. Advanced animal pharmacology was reported for AP-237 (36).[53,54]



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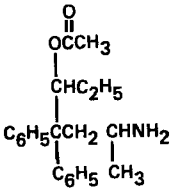

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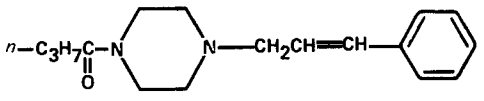

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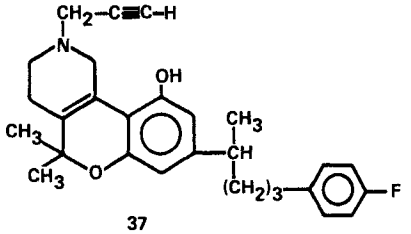
Cannabinoid Analgesics

The relative analgesic potencies of Δ^9-THC and codeine were evaluated in cancer patients.[55,56] While oral Δ^9-THC (10 and 20 mg) provided pain reduction equivalent to codeine (60 and 120 mg), the higher dose of Δ^9-THC produced marked sedation, limiting its usefulness. Contrary to an earlier report that smoking marijuana increases sensitivity to pain in experienced smokers,[57] a recent double blind study[58] showed a statistically significant increase in pain threshold after smoking marijuana. A greater increase in pain tolerance was observed for the experienced than for the non-experienced subjects. While Δ^9-THC caused minimal respiratory depression alone, it was found that the combination of Δ^9-THC and oxymorphone profoundly depressed ventilation in healthy volunteers.[59] Synthetic cannabinoid analgesic Abbott-41,988 (37) was demonstrated to be a potent oral analgesic in animals (= anileridine in mouse writhing and rat tail flick) having a slow onset and long duration of action.[60] Nabilone (38), currently undergoing clinical evaluation as an antianxiety agent,[61] also exhibited significant analgesic activity in animals.[62]

Details of the analgesic activity of Δ^8- and Δ^9-THC and their hydroxylated metabolites in animals were reported.[63] The analgesic effects of Δ^9-THC, cannabinol, and cannabidiol were compared with morphine and aspirin.[65] In the mouse writhing procedure, the following relative oral potencies were determined (oral

ED_{50}'s in mg/kg); Morphine (2.9) > Δ^9-THC (4.2) > cannabinol (35.0) > aspirin (130.0) ≫ cannabidiol (400.0). It was concluded that Δ^9-THC possesses morphine-like analgesic activity while cannabinol appears to be an aspirin-like analgesic. The report that naloxone antagonizes 11-hydroxy-Δ^8-THC analgesia suggests that these hydroxylated metabolites and morphine share a common mechanism for producing analgesia.[63] However, a definite conclusion that these hydroxylated derivatives act by an opiate-like mechanism is not possible since 9β-hydroxy-9-nor-hexahydrocannabinol does not bind to the opiate receptor.[64] Two reviews updating recent developments in cannabinol chemistry and pharmacology were published.[66,67]



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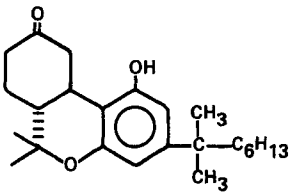

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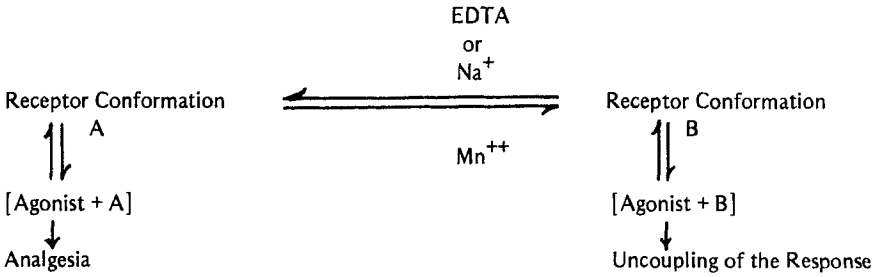
Biochemistry, Neurophysiology and Pharmacology

The Opiate Receptor – Regional opiate receptor binding has been found to vary significantly in different brain structures in the rat, monkey and human brain. The highest levels of binding have been found in the amygdala and the periaqueductal grey of the midbrain, hypothalamus and medial thalamus; regions of the brain which have been repeatedly associated with opiate activity and nociception.[1,68-70] Microscopically localized concentrations of receptor sites occur in the locus coeruleus and the zona compacta of the substantia nigra.[71,72] Thus, both noradrenergic and dopaminergic systems appear to be intimately associated with opiate receptor binding. Heavy labelling of the substantia gelatinosa in the lower brain stem and spinal cord also suggest involvement with major sensory tracts.[71,72] The autoradiographic distribution for the opiate receptor does not parallel that of acetylcholine or the biogenic amines.

Biochemical identification of the opiate receptor has also permitted direct examination of opiate receptor interactions. Binding has been observed in the guinea pig ileum[73,74] and in cell cultures of neuroblastoma x glioma hybrids[75,76] which parallels binding to rat brain. Binding has been closely correlated with the pharmacological activity of both agonists[77,78] and antagonists.[79] A requirement for chirality in the N-substituent of a series of benzomorphans (**18**) has been demonstrated.[30] Interestingly, diphenoxylate and loperamide show no selectivity for ileum over brain despite their apparent clinical selectivity as antidiarrheals.[73]

An allosteric model of the opiate receptor with two interconvertible forms has been proposed (see below).[80,81,82] In this model agonists and antagonists are postulated to act on different conformations of



the same receptor. The conformational change induced by Na ion facilitates antagonist binding,[1,85] protects the receptor against specific deactivation of the "A" conformation by protein modifying reagents,[81,83,84] and provides the best correlation between biological activity and binding affinity.[74] The divalent cation Mn, as well as Mg and Ni ions, are capable of reversing this Na^+ dependent increase in antagonist binding.[86] Experiments with chelating agents suggest that an unidentified endogenous divalent cation is normally involved in the regulation of receptor function. That agonists and antagonists act upon different conformations of the same site is supported a) by evidence that the same number of sites are found using either agonists or antagonists, b) that actions which inactivate the agonist site are blocked by both agonists and antagonists[81] and c) by the partial activity seen for the mixed agonist/antagonists in the neuroblastoma system.[82] The possible existence of further discrete and non-interconvertible receptors has also been proposed based on binding studies and pharmacological evidence.[87,88]

Neurophysiology – Morphine's major site of action has been localized in the periaqueductal central grey region of the brain stem. For example, activation of ascending pathways by pain, but not by stress, lowers glutamate levels in the periaqueductal central grey, and this decrease in glutamate concentrations is specifically antagonized by morphine.[90] Lesioning and stimulation experiments in animals have shown that morphine acts through, but not directly, on descending serotonergic pathways of the adjacent raphe nucleus.[91,92] This is postulated to produce an indirect depression of activity at the level of the dorsal horn neurons which are involved in the full elaboration of painful stimuli at the spinal level.[93,95] Morphine has also been shown to have some direct activity at the spinal level.[94] Striking similarities have been noted between the analgesia and tolerance produced by both morphine and by electrical stimulation of the central grey area,[89,97] This evidence of morphine-like analgesia produced by lanthanum ion[98] and the recent finding that acupuncture analgesia is reversed by naloxone[99] again argue for the presence of a novel neural system in the mammalian brain that utilizes an endogenous morphine-like factor to modulate pain perception.

Enkephalin – The endogenous morphine-like factor suggested by neurophysiological studies and work on the opiate receptor, has now been identified in the brains of a number of mammalian species[100-102] and in cerebrospinal fluid (CSF) in man.[103] Its regional distribution in rat and calf brain is remarkably similar to and well correlated with the regional distribution of the opiate receptor.[101] Enkephalin binds to the opiate receptor with an affinity comparable to that of morphine. Following intensive isolation work by a number of groups,[100-104] Hughes et al.[105] have now elegantly demonstrated that porcine enkephalin is composed of two pentapeptides, methionine-enkephalin and leucine-enkephalin in approximately a 3:1 ratio. Methionine-enkephalin is a potent agonist in the mouse vas deferens (20 x normorphine) and in the guinea pig ileum (1 x normorphine), activities which are completely reversed by naloxone. Leucine-enkephalin exhibits a similar

H–Tyr–Gly–Gly–Phe–Met–OH (methionine-enkephalin)

H-Tyr–Gly–Gly–Phe–Leu–OH (leucine-enkephalin)

profile of activities, but is somewhat less potent than its methionine cogener. Their structures have been confirmed by total synthesis and biological comparison with natural material.

Remaining to be shown is the relationship of the enkephalin peptides to similar peptides isolated by others[101] as well as to ACTH fragments[106] and to the substances isolated from bovine pituitary glands.[107] Of potential medicinal significance is the observation that enkephalin shares much of the unusual pharmacology of a series of benzomorphans (**18**) which are free of addictive properties in monkeys.[108] Synthesis of a heptapeptide based on theoretical considerations and on known opiate structure activity relationships has been reported.[109] Although this synthetic peptide is only slightly active, it represents a significant initial probe of the interface between peptide and classical drug moieties

The discovery of enkephalin and an associated neuronal tract will certainly have great significance for future research in the analgesic area. An intriguing indication of this is provided by the discovery that levels of a morphine-like factor are lower in the CSF of patients with trigeminal neuralgia than in patients without pain.[103] A further neurochemical role for enkephalin and related substances, extending beyond pain modulation, is suggested by its wide distribution in brain and its presence in guinea pig ileum.

Regulation of Adenylate Cyclase – Following the discovery of a prostaglandin E_1 (PGE) sensitive adenylate cyclase (AC) which is inhibited by morphine,[110] considerable attention has been focused on attempts to test the proposal that this system mediates opiate analgesia and dependence. While the originally described rat brain homogenate system has apparently proved difficult to reproduce,[111-112] opiate regulation of the AC system in a neuroblastoma x glioma hybrid cell line has been more reliable and has demonstrated an excellent correlation with *in vivo* data. In this system addition of morphine leads to rapid inhibition of basal and stimulated AC activity and to a consequent fall in cAMP levels. These actions are stereospecific, show an excellent correlation with opiate receptor binding affinities and are efficiently reversed by naloxone.[82,113] The degree of inhibition of PG stimulated AC activity correlates with the number of opiate receptors present in parental cell lines providing further evidence for mediation of the AC by opiates. Kinetic studies indicated that PGE and morphine act at closely related sites and exhibit cooperativity.[76]

Extended incubation of the neuroblastoma cell line with morphine reveals a compensatory increase in AC activity and a return to normal levels of cAMP.[114,115] In this biochemical equivalent of tolerance, morphine inhibition of AC leads to a compensatory rise in cAMP synthesis and hence, to a dependence of the cells on morphine for preservation of normal cAMP levels. Removal of morphine from these habituated cells causes an overproduction of cAMP, i.e. the biochemical equivalent of withdrawal. As is the case *in vivo*,[118] tolerance development in the AC system can be blocked by the protein synthesis inhibitor, cycloheximide.[115] In support of these findings increased brain levels of cAMP has been reported in abstinent rats[116] and a quasi abstinence syndrome has been seen following treatment with potent inhibitors of phosphodiesterase and naloxone.[117]

Opiates elevate intracellular levels of cGMP in the neuroblastoma system[115] suggesting that at least some of the effects of morphine on PGE stimulated cAMP formation may be mediated via cGMP. Morphine thus shares with α-adrenergic and cholinergic agonists a common feature – elevation of cGMP and reduction of cAMP levels[119] – and in fact, intracerebroventricular acetylcholine has recently been reported to produce morphine like analgesia.[120]

Involvement of Neurotransmitters – Increased dopamine and serotonin were generally found to facilitate analgesia in experiments involving both morphine and focal electrical stimulation, whereas norepinephrine appeared to have the opposite effect.[89] The well known increase in dopamine turnover produced by morphine was distinguished biochemically from increases elicited by antipsychotics, anticholinergics and amphetamine.[121] Pain related alterations in γ-aminobutyric acid (GABA)/glutamate ratios were antagonized by morphine, suggesting that morphine might control primary afferent neurons indirectly through an effect on GABA synthesis.[122] In line with this, baclofen, the 3-p-chlorophenyl analog of GABA, has been reported to possess analgesic activity.[123]

Tolerance and Dependence – Several comprehensive reviews of the neurochemical bases for narcotic dependence and tolerance appeared in 1975.[124,125,127] The bulk of new work has supported the hypothesis that development of tolerance to opiates involves a counteradaptive biochemical or neuronal change and not an alteration of the drug/receptor interaction. In line with this no significant changes were seen in the number or the binding affinity of receptors in tolerant mice.[126,128,129] Numerous studies have established that protein synthesis plays a requisite role in tolerance development.[118,128,129]

The applications of state-dependent learning to the evaluation of analgesics has been extended not only to the assessment of dependence liability, but also to the detection of side effects. For example, cyclazocine, which is known to possess psychotomimetic properties, was equated with LSD by rats trained to discriminate LSD from vehicle. In rats, the discriminative stimuli produced by morphine are blocked by naloxone and generalize to levorphanol and other opiates, but not to the inactive stereojsomer dextrorphan or to cyclazocine.[132-134] Despite their apparent analgesic specificity, the 9-nor-9-hydroxy hexahydrocannabinol analgesics described above were shown to generalize to Δ^9-THC in discrimination experiments.[131]

Screening Methodology – The guinea pig ileum and mouse vas deferens *in vitro* models have been used to predict accurately the relative antagonist activity of analgesics with dual agonist and antagonist actions.[135] Rates of onset and offset of action in the guinea pig ileum have been correlated with morphine-like dependence liability and lipid solubility.[136] A biphasic writhing syndrome induced by acetylcholine in mice has been used to differentiate between antiinflammatory and centrally-acting analgesics.[137] However, both writhing phases remained sensitive to potent CNS depressants such as chlorpromazine. Acetic acid-induced writhing in rats has been exhaustively studied and found to be a quantitative and sensitive method similar to phenylbenzoquinone writhing in mice.[138] The rat adjuvant-induced polyarthritis test has been adapted to predict accurately the relative analgesic activity of the narcotic narcotic-antagonist and antipyretic analgesics under conditions more relevant to chronic pathological pain in man.[139,140] A significant advantage of this model over other tests sensitive to the narcotic antagonist and antiinflammatory analgesics, is its reported ability to selectively exclude the potent CNS depressant drugs.

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Chapter 4. The Opiate Receptor

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This review covers the opiate receptor(s) and the endogenous analgetic substances, on which significant and rapidly developing progress has been made in the last few years. These subjects have been reviewed previously [1-5], but not in these volumes so a review with key references is believed to be appropriate. The work on the opiate receptor has been a landmark development in biochemistry, representing the first significant definition of receptor sites since the elucidation of the structure of hemoglobin.

The past few years have seen significant progress in the elucidation of the opiate receptor(s). During this period there was also elucidated the structure of endogenous hypothalamic and hypophyseal peptides of low molecular weight having the analgetic properties of morphine, including antagonism of its effects by naloxone. These researchers have also uncovered what may be a new neurotransmitter, and have provided some explanation of the biochemical basis of opiate addiction and tolerance. Finally, further significant progress in the separation of analgetic, tolerance and dependence producing properties has been effected by molecular modification.

Stereospecific binding of opiates to receptors (see also chapter 3) was discovered independently by four groups of investigators at Johns Hopkins University, at New York University, at Uppsala University and at Stanford [6-12], and was rapidly confirmed by a number of other investigators [12-15].

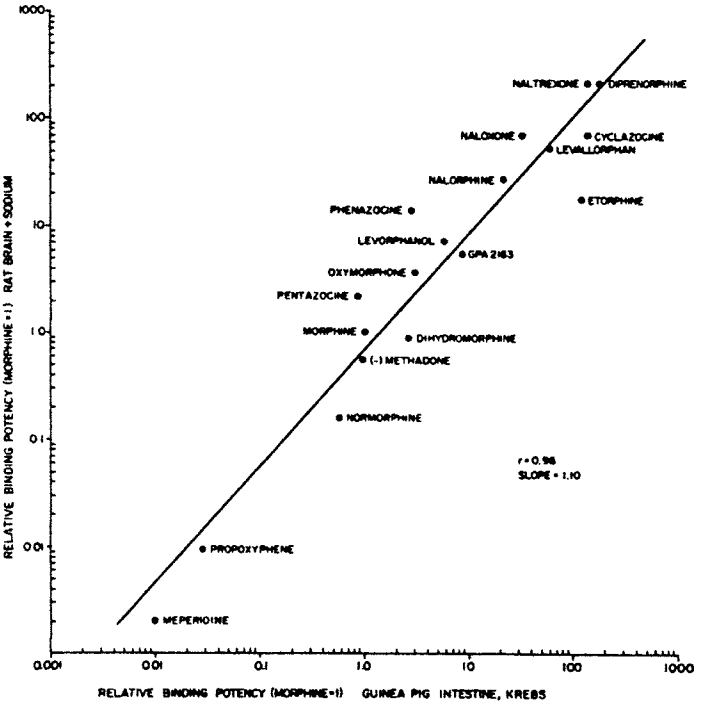
The receptor must function as both a ligand and as a mediator of a biological response. The criteria of receptors [17] may be said to be:

1) Specificity of agonist - both pharmacologic and steric, in particular a discrimination between d- and l-isomers of agonists and antagonists.

2) Saturability - implying that the number of receptors is limited.

3) Target cell specificity - binding should be observed to the same cells that exhibit a biological response.

It is of interest that Kosterlitz et al [19, 20] found that the affinity of opiates for the myenteric plexus of the guinea-pig ileum parallels brain affinity. Similar results were obtained by Kosterlitz with tissue mince and with homogenate preparations. Among subcellular fractions binding is greatest in the synaptosomal-mitochondrial fractions.



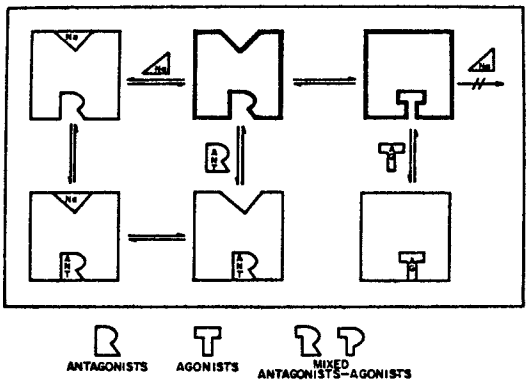
Correlation between the relative potencies of opiates to inhibit ^{3}H-naloxone binding in guinea pig intestine and rat brain homogenates.

From "Opiate Receptor Mechanisms," S. H. Snyder and S. Matthysse, eds., MIT Press 1975. Reprinted by permission of MIT Press.

Certain ubiquitous lipids like cerebrosides, (which occur in brain), and other acidic lipids (e.g. phosphatidic acid, phosphatidyl-inositol, triphosphoinositide, phosphatidylserine and ganglioside) can bind opiates stereospecifically [21, 22, 23, 60]. On the other hand, the binding of prostaglandins by opiate receptors is in dispute. Snyder [24] has commented on the glycine receptor as a model for the opiate receptor, noting the close correlation between endogenous levels of glycine, high affinity uptake into specific synaptosomes, the ability of glycine to mimic the natural inhibitory transmitter, and the density of the glycine receptor binding site.

Sodium markedly increases receptor binding of opiate antagonists and decreases to the same extent the binding of opiate agonists [25, 26]. This effect is highly specific for sodium and is seen to a lesser extent with lithium. Cesium, potassium and rubidium do not discriminate agonists and antagonists. Some divalent cations (Mg, Mn, Ni) produce an opposite effect to that of sodium [27]. Substances with mixed agonist-antagonist properties have appropriate intermediate sodium effects, while calcium depresses receptor binding of opiate antagonists but fails to enhance opiate agonist binding [28].

The temperature dependence of binding, among other things, suggests [26] that the opiate receptor exists in an equilibrium between two interconvertible forms, an antagonist form which is preferred in the presence of sodium and an agonist form which is preferred in the absence of sodium. Typical agonist or analgetic effects of opiates occur only if a drug binds to the agonist form of the receptor. Thus antagonists are said to bind to the sodium form of the receptor, shifting the equilibrium between the sodium and no sodium states and making fewer no sodium receptors available for interaction with agonists. Sodium thus has a crucial role in effecting the transition between the two states of the receptor [67].



A hypothetical model of the opiate receptor based upon classic allosteric drug-receptor models. The 2 heavily outlined figures represent the 2 interconverting receptor conformations that possess differential affinities for opiate antagonists and agonists.

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If the antagonist form of the receptor actually binds sodium while the agonist form does not, attachment of the agonist to the agonist conformation will produce a change (increase) in sodium concentration. Similarly, attachment of the antagonist to the antagonist conformation will produce an opposite change (decrease) in sodium concentration. It appears, therefore, that sodium is the ion responsible for changes in conductance produced by opiate agonists and antagonists.

Methods of accentuating specific opiate receptor binding and minimizing non-specific binding have involved use of small amounts of labeled opiates of high specific radioactivity followed by washing tissue rapidly to remove non-specific binding. In this way opiate receptors have been demonstrated in brain and intestine [10, 28, 29, 30]. Ideally, one should compare pharmacological activity and receptor binding in the same system, which is feasible because, as mentioned earlier [19, 20], the inhibition of electrically induced contractions of the guinea pig ileum by opiates closely parallels the analgetic activity of these opiates in the intact animal.

Pert et al [25, 26] have evaluated the influence of sodium on receptor interactions of a wide variety of opiate agonists and antagonists by measuring the extent to which sodium alters the ability of the drugs to inhibit receptor binding of tritiated naloxone. The resultant "sodium response ratio" is the ratio of the concentration of the drug which inhibits tritiated naloxone binding by 50% in the presence of sodium to the 50% inhibitory concentration in the absence of sodium. Thus a pure antagonist like naloxone has a ratio of one, while for a variety of opiate agonists the ratio is between 12 and 60. Mixed agonist-antagonists generally have a ratio in the 3-6 range. This technique permits characterizing potential agonist-antagonist analgetics with a few milligrams of material [5, 31, 24]. These results provide a rapid method for determining the potency and profile of an analgesic candidate.

That the opiate receptor had the general properties of a proteolipid was already suggested by the method of isolation [12]. The protein nature of the opiate receptor is further indicated by its great sensitivity to proteolytic enzymes [32] and sulfhydryl reagents [30]. Phospholipids apparently have no direct role in the binding as shown by the lack of effect of phospholipases [30]. It is of interest that low concentrations of protein-modifying reagents and proteolytic enzymes selectively reduce opiate agonist binding with negligible effects on antagonist binding [27, 32, 33].

Regional Mapping

Sharp pain sensations which can be discretely localized are conveyed by a laterally located sensory pathway in the ventrobasal thalamus, and in other regions. More chronic pain which cannot be attributed to a specific locus involves multisynaptic, slowly conducting, medially located pathways [34]. There are dramatic variations in opiate receptor

binding throughout the monkey and human brain [35, 36]. The highest binding area is the amygdala closely followed by the midbrain, hypothalamus, and medial thalamus. Receptor binding in the caudate nucleus is also high, and there are marked variations within the cerebral cortex. In the monkey, high binding sites are in the limbic cortex (amygdala and hippocampus), in the hypothalamus, in the medial thalamus, in the extrapyramidal area (caudate nucleus and putamen) and in the midbrain, superior colliculi, interpeduncular nucleus and periaqueductal gray area. In the human high binding sites this distribution of binding correlates with the areas in which implantation of morphine produces analgesia [37].

Opiate antagonists elicit withdrawal symptoms in addicted animals most markedly when implanted in the medial thalamus, which is also rich in opiate receptors [38].

It is interesting that the areas of highest binding of opiates are structures of the limbic system, which is involved in the perception of emotion and pain. Within the limbic system are found the pleasure (septal nuclei and preoptic hypothalamus) and punishment (amygdala, hypothalamus, thalamus and mid-brain) centers. The high level of receptor population in the limbic system is consistent with the two major effects of opiates, analgesia and euphoria.

It is possible to further pinpoint the precise location of opiate receptor sites with the aid of autoradiographic studies [39]. In intact animals [40] intravenous administration of tritiated diprenorphine (see chapter 3) results in more than 80% of the radioactivity in the brain being associated with opiate receptor sites [39]. For precise radioautography, fixing of tissues at very low temperatures prevents leakage of diprenorphine from receptor sites. An association of the effects of opiates on the metabolism of biogenic amines, particularly of catecholamines is possibly explained by the high density of opiate receptors in the locus coeruleus and zona compacta which consists almost exclusively of norepinephrine and respectively, dopamine cell bodies [41, 42]. Pert et al [65] have found, however, that even when the nerve tracts associated with the neurotransmitter systems (norepinephrine, acetylcholine or serotonin) are destroyed, opiate binding is unaffected, suggesting that opiate receptors do not operate through the established neurotransmitter systems.

Endogenous Morphine-like Factors

A clue to the existence of endogenous morphine-like factors was the work of Akil [63] who found that naloxone can antagonize the analgetic response to focal stimulation of the periaqueductal gray matter. It has been speculated that acupuncture may act through endogenous opiate and acupuncture induced analgesia is in fact reversed by naloxone [64].

A direct approach to identifying a possible neurotransmitter associated with the opiate receptor was adopted by Hughes [43], who found in

brain extracts a substance which mimics the ability of morphine to inhibit electrically induced contractions of smooth muscle preparations such as the mouse vas deferens or the guinea pig intestine. Furthermore Terenius and Wahlstrom [44, 62] and Pasternak et al [45] have reported that various brain extracts compete for opiate receptor binding. The morphine-like factor of Hughes, called "enkephalin" is a low molecular weight peptide, rapidly degraded by carboxypeptidase A and B, less rapidly by leucine aminopeptidase, and not by trypsin or chymotrypsin.

The molecular weight of Simantov and Snyder's peptide, called Endorphin [66], is also low, being composed of 7 or 8 amino acids with a molecular weight of about 1000, but the peptide obtained by Goldstein's group [46] from extracts of the pituitary glands is of much higher molecular weight. The low molecular weight peptides are degraded to a small extent by chymotrypsin, but not at all by trypsin [43, 45, 46]. In opiate binding assays the morphine-like factor behaves like an agonist, since its ability to compete for binding is impaired by sodium and protein-modifying reagents, but enhanced by manganese [45]. Its regional distribution in calf, rat, and rabbit brain is closely similar to that of the opiate receptor itself [43, 44, 45, 46].

Enkephalin, a mixture of 2 pentapeptides, methionine-enkephalin and leucine-enkephalin [47] (see chapter 3), induces a profound analgesia in rats in vivo that is fully reversible by naloxone [18].

Since the principal sequence in methionine-enkephalin is identical to that of residue 61-65 of beta lipotropin, a peptide found in porcine-pituitary, in vivo protelolytic cleavage of the C fragment was suggested for the biosynthetic origin of methionine-enkephalin [48,69]. It was also suggested that there are conformational similarities between methionine-enkephalin, morphine and oripavine [48].

A chemically different peptide, which also mimics the effect of morphine to smooth muscle has been isolated from the pituitary [2]. The morphine-like factor has been thought to be an inhibitory transmitter [49].

Teschemacher et al [2] have reported further that the morphine-like substance from bovine pituitary glands inhibits electrically stimulated mouse vas deferens and guinea pig intestine. This inhibition is reversed and blocked by naloxone. The extract also inhibits binding of opiate agonists and antagonists to stereospecific sites in the synaptic membranes of guinea pig brain. The inhibition of naloxone binding is decreased by sodium ion in the manner characteristic of the opiate antagonists. The physiological role of the pituitary opioid remains to be investigated.

A number of other synthetic samples of peptides that occur naturally in the pituitary or hypothalamus were also tested for opioid properties. Included were synthetic human ACTH, α-MSH, tetracosactin, lysine-vasopressin, oxytocin, luteinizing hormone releasing hormone (LHRH), substance P, Pro-Leu-Gly - NH_2(MSH-RIH) and somatostatin. Somatostatin

produced an inhibition of the electrically induced contraction of the guinea pig ileum, an effect which could not be blocked by naloxone. The rest of the synthetic peptides were ineffective. A crude porcine ACTH, however, gave a positive result both in the guinea pig ileum and in the opiate receptor binding assay developed by Pert and Snyder and by Simon et al [30]. From the crude porcine ACTH an active opioid component was isolated. The active opioid contaminant in crude ACTH preparations is similar to the principle isolated from bovine pituitary [50].

Following the discovery that some endogenous peptides exert opioid-like effects the synthesis of an opioid-like heptapeptide has been reported [51]. The synthesis of the heptapeptide was based on the theory that proper positioning of aminoacids should match the essential features of opioids in three-dimensional space. The linear heptapeptide with the structure of H-Tyr-Gly-Gly-Gly-Lys-Met-Gly-OH produced opiate-like effects in the guinea pig ileum and in the opiate receptor binding assay. It was proposed [51] that the linear peptide takes up an α-helix conformation in order to bring the tyrosine and lysine portions close enough in space, and that such a conformation is necessary for opiate-like action.

An analogy was drawn between hypersensitivity to antagonists contrasted to subsensitivity to agonists observed in the state of addiction on one hand and the properties of the sodium or antagonist state of the opiate receptor as contrasted to the no sodium or agonist state on the other hand. The theory has been put forward that tolerance and physical dependence can be correlated with changes in the opiate receptor which may be less capable of assuming the agonist form, favoring instead the antagonist, sodium form [25, 26]. The greater sensitivity of dependent subjects to antagonists is consistent with the hypothesis that the opiate receptor exists in two forms, but the subject is still open to other hypotheses.

It was suggested that morphine and morphine-like substances (e.g. the endogenous factor) act as an inhibitor of prostaglandin-stimulated adenylate cyclase mechanism [52]. Morphine and related substances block the enhanced formation of cyclic AMP caused by addition of prostaglandin E to homogenates of rat brain. It is possible that like numerous hormones and neurotransmitters the endogenous morphine-like factor may have as its second messenger a cyclic nucleotide although this suggestion is still controversial. In addition to cyclic AMP, cyclic GMP has also been suggested for such a role [53, 54, 55, 56, 57, 58, 59, 61]. (see chapter 3).

Klee [68] has demonstrated that long-term exposure to opiates may produce a compensatory adenylate cyclase activity, and this late positive regulation can account for the tolerance and dependence phenomena of opiate addiction.

The isolation and identification of the endogenous opiate-like factor(s) is a milestone in the analgetic saga. Thus the rapid developments in this chapter will be followed closely by those seeking a

rational approach to the design of new analgetic agents.

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Chapter 5. Biological Factors in Psychiatric Disorders

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Introduction - A number of recent reviews[1-8], including one in a previous volume of this series[1], have presented comprehensive statements or critiques of the evidence indicating interactions between biological and psychosocial factors in the etiology of the major psychoses and other psychiatric disorders. This chapter provides a more circumscribed listing and brief commentary on research developments over the last year in the area of biological studies of these disorders. It highlights fields of current interest, but makes no attempt to integrate the findings into a balanced schema involving the many possible etiologic factors in these complex disorders.

Diagnostic Considerations and Genetic Studies - All clinical studies, including those of biological factors, rest upon accurate, reliable and valid diagnostic assessment. Adequate diagnostic reliability has been demonstrated when standardized assessment scales are used, of which there are several new or refined versions available[9-12]. However, the typical clinical syndrome descriptively cataloged using such scales may or may not represent a disease entity. Sharply delimited criteria for schizophrenia may yield a more homogenous population for research, but as one study indicated[13], may exclude as much as 88% of a hospital population receiving a clinical diagnosis of schizophrenia. The higher incidences of disorders other than schizophrenia itself, such as the schizophrenia spectrum disorders[14] or even affective disorders[15], in the relatives of schizophrenic patients, pose further problems for clinical and genetic studies.

Many models have been proposed for interpretation of the familial occurrence of psychopathology, but none satisfy all available data. Continuing developments in genetic studies of schizophrenia have been recently reviewed[16]. Single major locus or multifactorial models do not fit the population incidence, family and twin data when it is comparatively examined; one suggested possibility is a model with two interacting genes, although approximations using all models predict genetic heterogeneity among schizophrenic individuals[16]. Among the affective disorders, the utilization of the bipolar/non-bipolar dichotomy has aided in the investigation of the more homogenous bipolar subgroup, and has led to several studies strongly supporting X-linkage as one form of transmission of the bipolar disorder[17,18]. Not all bipolar families follow the X-linkage pattern, however, and a threshold model for the affective disorders has also been developed[19].

Neuroendocrine Responses

Growth Hormone and Prolactin in Patients with Affective Disorders and Schizophrenia - The neuroendocrine status of patients with depression has been assessed by measurements of the plasma growth hormone increase in response to insulin and L-dopa[20-23]. Unipolar postmenopausal depressed

women have reduced growth hormone responses to insulin in comparison to normal postmenopausal women[22]. Two earlier reports indicating reduced growth hormone levels in depressed patients in response to L-dopa by the same research group[20,21] were not substantiated when more appropriate control groups were studied[23]. Baseline serum prolactin levels were no different in schizophrenic patients compared to controls; prolactin elevations occurred in all patients in response to treatment with chlorpromazine, thioridazine and trifluoperazine, and remained elevated throughout the period of treatment[24].

Thyrotropin-Releasing Hormone (TRH) - This peptide has been reported to have antidepressant effects when given intravenously[25,26]. Thyrotropin (TSH) responses to TRH were also reduced in depressed patients, but returned to normal with recovery[27]. Studies in more severely depressed patients treated with both intravenous (600-1000 µg/day) and oral (40 mg/day) preparations did not confirm an antidepressant effect[28-30]. Larger oral doses (300 mg/day) were associated with deterioration in the clinical status of seven out of nine male schizophrenic patients, particularly patients with paranoid symptoms[31]. As mild stimulant responses were the predominant change noted in normal volunteers given TRH, it has been suggested that TRH might possess some amphetamine-like effects[31], which would be consistent with its ability to potentiate L-dopa induced stereotyped behavior in mice[32].

Enzyme Studies

Creatine Phosphokinase and Neuromuscular Abnormalities - The elevations in serum creatine phosphokinase (CPK) regularly observed in patients with acute schizophrenia and affective psychoses[33] and upon exercise stress in patients in remission[34] are thought to result from changes in skeletal muscle. Cerebrospinal fluid levels of the enzyme are not increased, and the serum elevations are of the muscle CPK isozyme. Possibly related abnormalities in the innervation patterns of skeletal muscle in psychotic patients have recently been described[35], consisting primarily of increased subterminal branching together with the presence of immature branches. Resultant neurogenic motor abnormalities might help explain the observations of eye-tracking abnormalities and other fine motor control problems in schizophrenic individuals.

Catechol-O-Methyl Transferase (COMT) - This degradatory enzyme for norepinephrine, epinephrine and dopamine has been measured in soluble and membrane fractions from human erythrocytes in patients with psychiatric disorders. It was originally reported as reduced in activity in women with primary affective disorders, particularly non-bipolar patients[36-38], although not all studies are in agreement[39-41]. A recent study of affective disorder patients and their relatives in Israel found significantly higher enzyme activity in both female and male patients[41]. Non-bipolar patients tended to have higher activities than bipolar patients, and females had higher COMT activities than males. Sib-sib and mid-parent to mid-offspring activities were significantly correlated, a finding compatible with a genetic determination of enzyme activity

differences[41]. Within families, ill relatives and patients had higher activities than recovered patients. On the basis of the findings from this recent study, erythrocyte COMT has been suggested to be a possible marker for vulnerability to affective disorders[41].

Monoamine Oxidase (MAO) - Reductions in platelet MAO activity have been observed in most studies of patients with chronic but not acute schizophrenia, and in patients with bipolar but not unipolar affective disorders[42,43]. Genetic factors seem to be the single largest contributor to individual differences in enzyme activity, although sex steroids may also contribute, as small activity changes occur during the menstrual cycle, and most studies have reported higher activities in females[42].

Brain MAO activity measured in postmortem samples was not different in schizophrenic patients[44-47], although only one study[46] has examined multiple substrates, and none has examined specific inhibitors of the enzyme in conjunction with multiple substrates -- a consideration since brain contains predominantly the A form of MAO together with some B form, while the platelet contains essentially only the MAO-B[48]. Two reports have identified substrate-specific MAO activity differences in schizophrenic patients compared to controls[49,50], and the existence of a molecularly-different MAO form in schizophrenic patients has been suggested[49].

Brain MAO activity measured with both β-phenylethylamine and tryptamine was found to be reduced an average of 28% in all 13 brain regions studied in 15 suicides compared to 20 controls[51]. Non-significant MAO reductions of 13% in four depressive suicides and of 18% in three alcoholic suicides compared to seven controls were reported in a study using kynuramine as substrate[52].

Studies of rhesus monkey populations have provided more evidence that sex steroid changes contribute to MAO activity differences, and have also documented sex-specific behavioral correlates of individual differences in MAO activity in such dimensions as activity and social contact behaviors[53]. Normal college students have also been found to have MMPI and other personality profile correlates with individual differences in platelet MAO activity[54].

Other Enzymes - Significantly higher (+ 29%) erythrocyte membrane Mg^{++}-dependent adenosine triphosphatase activity was found in schizophrenic patients compared to controls[55]. Reduced (- 19%) sodium pump activity (ouabain-inhibitable Na^+ extrusion from cells preloaded with Na^+) was present in erythrocytes from patients with bipolar affective disorders[56]. Mucosal potential differences, however, measured in patients with recurrent affective disorders and controls were no different[57]. Serum indolethylamine-N-methyltransferase activity was not different in schizophrenic patients compared to controls, although one of two paranoid subgroups had higher enzyme activities, and there was a positive correlation between enzyme activity and severity of delusions[58].

Biogenic Amines and Their Metabolites

Cerebrospinal Fluid 5-HIAA, HVA and MHPG in Patients with Psychiatric Disorders - As reviewed recently by Goodwin and Post[59], cerebrospinal fluid levels of the serotonin metabolite, 5-hydroxyindoleacetic acid (5-HIAA) have been reported as significantly lower in five of ten studies of depressed patients compared to controls. Reduced levels of the dopamine metabolite, homovanillic acid (HVA) have been observed in four of six studies of depressed individuals. The norepinephrine metabolite in cerebrospinal fluid, 3-methoxy, 4-hydroxy phenyl glycol, was lower than controls in only one of three studies, although the study reporting the difference had the largest number of subjects and controls. Difficulties in obtaining controls, as well as complicating variables which can affect amine metabolite levels in cerebrospinal fluid (e.g. physical activity) may have contributed to some of the variability in results. The use of probenecid to block transport of the acid metabolite out of the cerebrospinal fluid has enhanced the differences between depressed patients and controls, with four out of five of such studies reporting reductions in both 5-HIAA and HVA[59]. Treatment with tricyclic and MAO-inhibiting antidepressants and with lithium all lead to reductions in 5-HIAA levels in the cerebrospinal fluid. In addition, there is some evidence for the persistence of reduced 5-HIAA and MHPG levels in depressed patients who are studied after clinical recovery[59].

Acute schizophrenic patients were not different from controls or from other psychiatric patients in baseline or post-probenecid CSF levels of 5-HIAA or HVA in a recent report[60]. These findings are similar to those from most of the nine previous studies reviewed; lower 5-HIAA values had been observed in two earlier studies and lower HVA in one earlier study. In this most recent report[60], HVA levels were lower after recovery from acute psychosis in patients studied in both acute and recovery periods. Baseline MHPG levels were measured in a smaller number of acute schizophrenic patients and were no different from controls[41].

Dopamine and Schizophrenia - The hypothesis that the schizophrenic syndrome might be explained, in part, by an overactive central dopaminergic neurotransmitter system continues to be a major stimulus to research[61]. The indirect evidence from the high correlations between the clinical efficacy of the antipsychotic phenothiazines and butyrophenones and their blockade of both the dopamine-stimulated adenyl cyclase[62,63] and also dopamine release from presynaptic sites[64] has strengthened the suggestions that these drugs act via dopaminergic mechanisms. The production of the symptoms of a paranoid schizophrenic syndrome in normals and the exacerbations of psychotic symptoms in schizophrenic patients in partial remission by methylphenidate, amphetamine and, in some instances, L-dopa also suggest that dopamine may be involved in such clinical states[61,65].

Nonetheless, the studies of homovanillic acid in cerebrospinal fluid[60], and of central neuroendocrine responses mediated by dopamine[24], as well as of dopamine-stimulated adenyl cyclase from postmortem brain samples[66] have not revealed any direct evidence of an alteration in

dopaminergic function in schizophrenic patients. It has been suggested that a functional dopaminergic hyperactivity state could result from a deficiency in a dopamine-balancing neurotransmitter system, such as in the GABA or acetylcholine pathways[24]. There is also a body of data implicating dopamine-related hyperactivity to mania as well as to schizophrenia, a view strengthened by evidence that lithium treatment is associated with a partial blockade of the activation and euphoria responses to d- and l-amphetamine[67].

Phenylethylamine, Tyramine, Octopamine and Dimethyltryptamine - Phenylethylamine is synthesized in human brain and other mammalian tissues by the decarboxylation of phenylalanine. Its stimulant effects on motor activity and exploratory behavior, which are enhanced by MAO inhibitor pretreatment, has led to suggestions of its possible involvement in the depressive disorders[68-70], with some supportive data coming from the measurement of urinary phenylethylamine levels in depressed patients[70]. It has also been hypothesized that, as an endogenous compound with amphetamine-like actions, it might contribute to schizophrenic symptoms[71]. Phenylethylamine[72] and tyramine[73] have also been suggested as triggering substances for migraine and seizures, respectively. Octopamine has been found in human brain[74] and platelets[75]. Its "false transmitter" effects have been considered as potential contributors to depressive symptoms[76] and heptic encephalopathy[77,78]. The indole hallucinogen, dimethyltryptamine, was present in the urine of more patients with schizophrenia and non-schizophrenic psychoses than of patients with other psychiatric disorders or controls[79].

Neurotransmitter Receptor Function - Following the development of techniques for identifying and quantitatively studying brain neurotransmitter receptors[80], there has been increased interest in primary or adaptational changes in receptor function in psychopathological states. Some strategies for studying receptor function in man have been reviewed[81]. Supersensitivity in dopamine receptors is one potential mechanism for the postulated dopaminergic hyperactivity in schizophrenia, and it has been observed after the discontinuation of phenothiazines[82]. Two different dopamine receptors[83] and/or the existence of dopamine receptors subserving different functions[84] may aid in explaining discrepant findings. As noted above, CSF metabolite, neuroendocrine and adenyl cyclase studies have not provided direct evidence supportive of a dopamine receptor alteration in schizophrenia. Similarly, attempts to evaluate suggested receptor function alterations in depressed patients[85] using adenyl cyclase responses to agonists have identified changes in response to drugs like lithium but have not found differences in patients compared to controls[86,87].

Other Studies

Eye-tracking and Psychopathology - Smooth pursuit eye movements examined by electro-ocular recordings during a pendulum tracking task are impaired in schizophrenic patients[88,89]. These studies have generated special interest because non-ill relatives of schizophrenic patients also show

this abnormality, and it has been suggested that this characteristic may represent a psychophysiologic marker for schizophrenia[88,90]. Patients with affective disorders, however, also have a similar impairment[89]. An attentional element has been implicated on the basis of improvement produced by requiring patients and normals to read numbers affixed to the pendulum[91], although alerting instructions alone had no effect[89,90]. While poorest performers improved most with the attentional task addition, patient groups were still significantly different from controls[91]. Abnormalities in extraocular muscles, the retina, central neural control mechanisms and involuntary attentional deficits have been suggested as possible contributors to the impairment on this test verified in patients and their relatives[88-91].

Additional Investigations - Schizophrenic behavior was exacerbated by the administration of wheat gluten, particularly in more severe, treatment-resistant patients[92] supporting earlier studies suggesting that cereal grains may be pathogenic in schizophrenia, and that there may be some relationship between schizophrenia and celiac disease[93]. Histochemically-studied leukocytes from schizophrenic patients uniformly showed an altered chromatin ultrastructure[94].

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Section II - Pharmacodynamic Agents

Editor: John E. Francis, CIBA-GEIGY Corp., Ardsley, N.Y.

Chapter 6. Pulmonary and Anti-allergy Drugs

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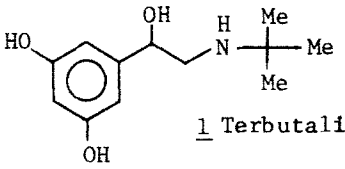
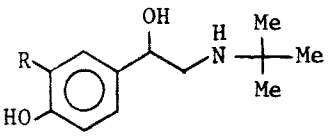
General - Allergy may be defined as an adverse response to a stimulus that provokes no untoward reactions in most individuals. There are two broad categories of allergic reaction; immediate and delayed hypersensitivity. Delayed responses include allergies to poison ivy, drugs, chemicals, etc., while immediate hypersensitivity includes syndromes such as allergic rhinitis, uticaria and asthma.[1-3] Bronchial asthma represents a major therapeutic problem since 10 million persons in the U.S. suffer symptomatology associated with asthma[4] and it results in perhaps 4,000 deaths annually. Typically, asthma is characterized by bronchial wall edema, reversible bronchospasm leading to paroxysmal dyspnea and wheezing.[5] Bronchial asthma may be classified into intrinsic (not clearly associated with a known immunologic mechanism) and extrinsic or allergic asthma which may be mediated by the union of antigen with specialized immunoglobulins (IgE).[6] IgE antibodies associate with host basophils and mast cells and when a specific antigen is encountered, mediators are released into the extracellular space. These mediators modify effector structures giving rise to clinical syndromes termed Type I immediate hypersensitivity.[7] In Type III, or delayed onset asthma, IgG antibodies may form aggregates with specific antigens, fix complement and provoke an inflammatory response (4-8 hr. onset).[8] Other theories concerning the pathogenesis of extrinsic asthma have been advanced, including decreased β-adrenoreceptor activity,[9] increased α-adrenergic activity[10] and changes in reflex cholinergic mechanisms.[11] Possible therapy may be divided into the following categories. (i) direct antagonists of chemical mediators, e.g. slow reacting substance of anaphylaxis, eosinophil chemotactic factor of anaphylaxis, neutrophil chemotactic factor, prostaglandins, histamine, basophil kallikrein, bradykinin;[12] (ii) β-adrenergic stimulants; (iii) anticholinergics; (iv) phosphodiesterase inhibitors; (v) steroids; (vi) prostaglandins; and (vii) inhibitors of mediator release (prophylaxis).

Beta-Adrenoreceptor Stimulants - A review regarding relative efficacy, duration of action and toxicity of the β-adrenergic bronchodilator aerosols used in treatment of bronchial asthma has been published.[13] The investigation of compounds claimed to be specific β-2 receptor stimulants (mediating bronchodilatation) continues. The goal is an orally effective compound with a long duration of action.[14] Terbutaline (1) has now been introduced onto the U.S. market. It is effective orally[15], intramuscularly[16] and by inhalation.[17] Inhalation of 1 is characterized by rapid onset of action (5 min.) Orally, peak effect is at 2-3 hrs., and significant bronchodilatation is evident for 3-7 hrs.[13] Pharmacokinetics of biliary excretion of intraarterially administered 1 to unanesthetized rats with uninterrupted



1 Terbutaline

enterohepatic circulation has been described.[18] Minimal CNS side effects (palpitations and trembling) have been noted.[19] This may be expected from the effect on (i) soleus muscle (ii) heart rate and (iii) airway resistance studies in cats.[20]

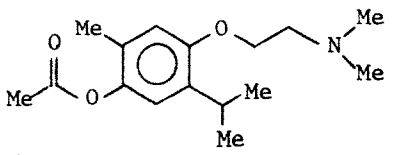


2 R = CH_2OH; Salbutamol

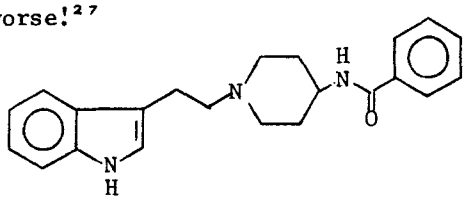
3 R = CH_2SO_2Me; (SKF 53705A)

A double blind study using salbutamol (2) showed efficacy in the inhibition of exercise induced bronchospasm in children[21] and in "status asthmaticus" 2 (300 µg/i.v.) increased pulmonary expired force (PEF) by 44%.[22] Of a series of catecholamine analogs bearing a substituted sulfonyl or sulfonylalkyl group on the meta position, sulfonterol (SKF 53705A, racemate) 3 showed enhanced selectivity for airway smooth muscle receptors. This, coupled with its lessened propensity to decrease soleus muscle tension, were the basis of its selection for clinical trial as a bronchodilator.[23] Structure-activity relationships show length and branching of the alkylene bridge joining the substituted sulfonyl group to the aromatic ring follow the order $CH_2 \gg (CH_2)_2 > (CH_2)_3 \simeq CH(Me) \gg$ no alkylene bridge. Upon substitution of the sulfonyl group, β-stimulant agonist potency decreased as the bulk of the substituent increased.[23] SKF 53705A (3) was slightly less potent than isoproterenol and salbutamol in relaxing guinea pig tracheal smooth muscle but several orders of magnitude less potent in increasing rate and force of guinea pig atrial smooth muscle.

α-Adrenoreceptor Antagonists - Although α-adrenoreceptor antagonists such as thymoxamine (4) and indoramine (5) have no inherent bronchodilator properties, administration in combination with a β-adrenoreceptor stimulant has been shown to produce beneficial effects. A combination of thymoxamine, or indoramine and salbutamol was effective in patients previously unresponsive to salbutamol alone.[24-26] However, a combination of indoramine and practolol appeared to make bronchospasm worse![27]



4 thymoxamine

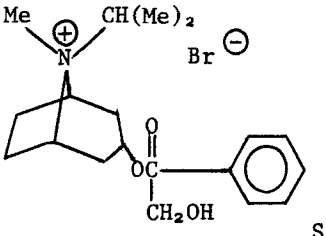


5 indoramine

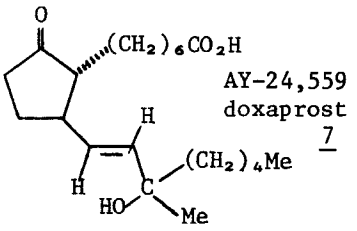
In conclusion, bronchial muscle tone is controlled by the autonomic nervous system with sympathetic and parasympathetic stimuli exerting opposite effects. Recent evidence[28] suggests that the parasympathetic nervous system plays a primary role in amplifying mediator effects secondary to antigen-antibody induced mediator release. Therefore, use of drugs which

affect α and β adrenergic systems, either alone or in combination, may provide greater therapeutic value and increased selectivity in the treatment of asthmatic bronchospasm.

Anti-cholinergics - Although the exact role of anticholinergics in the therapy of asthma has not been defined, numerous reports[29-31] show that inhalation of ipratropium bromide (Sch 1000) (6) produced bronchodilation similar to that achieved with salbutamol and isoproterenol.



Sch 1000 6



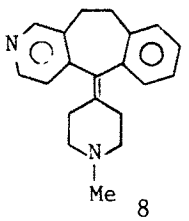
AY-24,559
doxaprost
7

Prostaglandins - A review concerning the clinical relevance of prostaglandins with respect to pulmonary physiology has appeared.[32] There are a number of advantages to the potential use of prostaglandins (PGE_1 and PGE_2) as bronchodilators. PGE_1 and PGE_2 are natural constituents of lung tissue and are potent agents in the rat passive cutaneous anaphylaxis (PCA) test. They show less side effects after aerosol administration than systemic administration because of virtually complete and rapid inactivation at the site of action. Both PGE_1 and PGE_2 increase intracellular cAMP and thereby inhibit mediator release which may account for an additional anti-allergic effect.[33-34] Clinical studies[35] have shown that aerosol administration of PGE_1 and PGE_2 were 10 times more effective than isoproterenol in bronchodilation ability in patients with reversible airways obstruction. They exhibited a slower onset and longer duration of action than isoproterenol. The major side effects appear to be pharyngeal irritation, laryngospasm, and cough. Another major disadvantage is their relative instability in solution. In contrast $PGF_{2\alpha}$ induced severe bronchoconstriction which was not inhibited by disodium cromoglycate (DSCG).[36] The results of a study in which atropine, DSCG and thymoxamine were tested against $PGF_{2\alpha}$ induced bronchoconstriction in extrinsic asthma suggests that locally formed $PGF_{2\alpha}$ may not be the main factor in the pathogenesis of bronchial asthma.[37] It has been proposed that the relative rates of PGE:PGF synthesis may account for the bronchospasm seen in bronchial asthma.[38] However, the use of generalized prostaglandin synthetase inhibitors, e.g. aspirin, indomethacin as anti-asthmatic drugs has proven ineffective.[39]

A study of the effect of PGE and PGF on airway conductance in healthy and asthmatic subjects showed that whereas 50% reduction of specific airway conductance was obtained in healthy volunteers with doses of up to 1 mg $PGF_{2\alpha}$, a dose of .5 mg or less was required to cause an 80% reduction in asthmatic subjects.[40] Also, PGE_2 was found to be a bronchodilator in healthy subjects but may cause either bronchodilation or bronchoconstriction in asthmatics.[40]

AY 24,559 (doxaprost) (7) is in clinical trial as a bronchodilator.[41]

Bronchodilators (other than β-adrenoreceptor stimulants) - Azatidine (8) has shown clinical efficacy and is a potent antihistaminic and antianaphylactic agent. In a closely related series of isomeric azaxanthenes, 9 is an orally effective bronchodilator with moderate antihistaminic activity.



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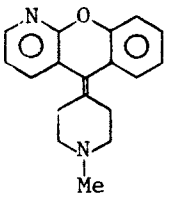

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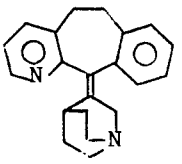
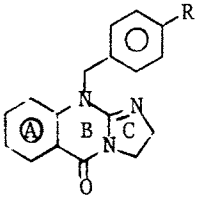


10

The azaxanthene 9 is not a β-adrenergic activator, but inhibits phosphodiesterase and the release of histamine from mast cells. Structure-activity relationships in this series show that the following cause loss of activity (i) movement of the N to other positions, (ii) tertiary carbinol formation (iii) movement of the double bond to an endo position and (iv) open chain versions of the piperylidine ring. Moderate activity is retained when the double bond is reduced, or when on the N-substituent of the piperidine ring Et replaces Me, or when the azaxanthene bears a fluoro substituent in the 7 position.[42] The related compound 10 only shows antihistaminic activity and the quinuclidine compounds are generally more toxic than the corresponding piperylidenes.[43] Structure-activity relationships have been published for a series of 2,3-dihydroimidazo(2,1-b)quinazolin-5 (10 H)-ones which are orally effective antagonists of histamine-induced bronchospasms. 11a is about 10 times as potent as theophylline while 11b appears to be 5 times the potency of theophylline. In the guinea pig anaphylaxis model 11a is claimed to be more potent than theophylline with no CNS or cardiovascular side effects. Modifications of the size of ring C showed the 5-membered ring compound to be more active than the 6-or 7-membered analogs. In ring B on the N substituent the activity followed the order benzyl⟩⟩ alkyl, allyl, propargyl⟩ phenyl. Ring A substitution yielded variable results.[44]



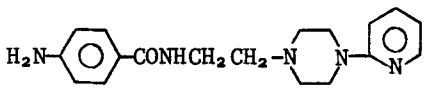
11a R=F

11b R=H

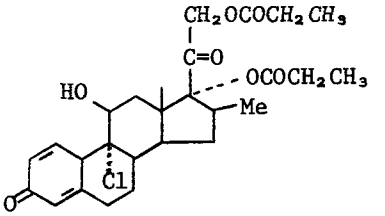
A procainamide derivative (S 1688) 12 provided protection intraperitoneally against histamine-induced bronchospasm in guinea pigs. An i.v. dose of 1-5 mg/kg in dogs caused long term hypotension, and postural hypotension was observed following aerosol administration in patients. Structure-activity relationships published indicate that changes anywhere in the molecule lead to greatly reduced activity.[45] S 1688 is an antihistaminic and antianaphylactic on intraperitoneal administration.

Corticosteroids - Beclomethasone dipropionate (13) aerosol was introduced into the U.K. market in 1972 and is presently in clinical trials in the U.S. It is considered a major advance in the treatment of asthma[47-51] and other reversible airways blockage diseases, particularly in children. Its chief

advantage is high topical activity (daily dose of 400 μg inhaled is equivalent to 7.5 mg prednisone p.o.)[52] together with low systemic activity due to metabolic inactivation of the swallowed portion of the dose. The most common side effect is a dose-dependent oropharyngeal candiasis.[53] Triamcinnolone acetonide aerosol (0.8 mg per day) was also shown to exhibit substantial symptomatic improvements in asthmatic patients despite a reduction of oral steroids. Side effects were a transient hoarseness and change of voice.[54,55]

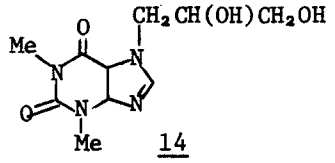


S 1688 12

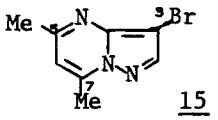


13 beclomethasone dipropionate

Phosphodiesterase Inhibitors - Pharmacokinetics of dihydroxypropyltheophylline (dyphylline) 14 by intramuscular and by oral administration (10 mg/kg) indicate a half-life of 2 hrs. and ready absorption independent of route.[56] A combination of theophylline-ephedrine has been shown to be no more effective than theophylline alone, and also to cause GI side effects, insomnia and restlessness not found with the same dose of the individual drugs.[57] The results of a regression analysis on a series of compounds related to ICN 3009 (15) (a cardiac selective PDE inhibitor) indicate that variation of the substituent at position 7 should lead to optimum cardioselectivity, and modification of the 5 substituent should lead to an optimally active lung PDE inhibitor.[58]

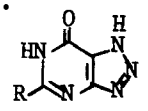


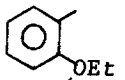
14



15

Inhibitors of Mediator Release - M & B 22,948 (16) (triethanolamine salt) was 40 times as potent as DSCG in the rat PCA model. The activity of a series of derivatives was correlated to the size and hydrogen bonding capacity of the ortho substituent in the phenyl ring.[59] Of a series of heterocyclic analogs, the best was 17, which in the rat PCA was 2.5 times as potent as DSCG on i.v. administration.[60]



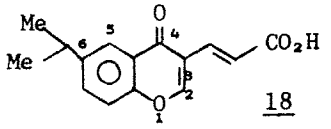
16 R = 

17 R = 

DSCG has now been on the market in the U.S. since 1973 and a comprehensive review of mode of action, pharmacology, therapeutic efficacy and use has been published.[61] There is still no clear indication how to select patients who will benefit most, but there is a trend towards more frequent response in (a) the young, (b) exercise-induced asthmatics and (c) patients with strong evidence of allergy dominant causative factor.[61] The bronchodilator and corticosteroid-sparing effect is one of the most important

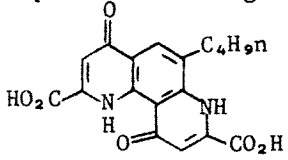
attributes of DSCG.[62] Reported side effects of DSCG are mild and infrequent (transient nasal congestion and throat irritation).[63] DSCG has been shown to induce hypersensitivity reactions in several patients. Those with clinically apparent adverse reactions produced lymphocyte migratory inhibitory factor (MIF) and serum binding activity for the drug.[64] The exact mode of action of DSCG has not yet been established but ^{3}H-DSCG is taken up by rat mast cells rapidly and lost equally rapidly (0.5 - 1 min.) suggesting that it may act at or near the surface of the mast cell membrane.[65] Clinical efficacy of DSCG does not appear to depend on modulating cell-mediated immune responses.[66]

Structure-activity relationships for a series of compounds in which 18 was the most potent intraveneously in rat PCA have been published. Efficacy by oral administration was also claimed for 18. The structural requirements for activity are (i) a trans double bond, (ii) an acrylic group at the 3-position of the chromone nucleus, (iii) alkyl or alkoxy at position 6. Other modifications result in loss of activity.[67]

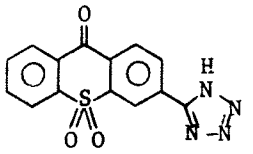


18

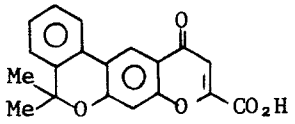
A tricyclic quinolone acid (ICI 74,917) (bufrolin, 19) is a potent inhibitor of certain types of immediate hypersensitivity. It exhibits anti-allergic properties additional to those possessed by DSCG in that it is effective in laboratory species other than the rat. Bufrolin, administered as the sodium salt (i.v.) was 300 times DSCG (rat PCA) and also active in mice and guinea pigs. In normal guinea pigs and in isolated lung preparation 19 provoked a mild bronchospasm. However, bronchospasm does not occur in seven other species including man.[64] 19 also shows cross reactivity with DSCG.[69]



19 ICI 74,917

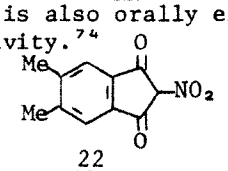


20 doxantrazole



21 PR-D-92-EA

A thioxanthene derivative, doxantrazole (20) suppresses PCA and allergic bronchoconstriction in rats. An oral dose of 200 mg inhibited the immediate type asthmatic response in 8 asthmatic patients challenged with a specific antigen, e.g. house dust mite.[70,71] The benzopyranobenzopyran carboxylic acid PR-D-92-EA (21) administered as the ethanolamine salt is very active in rat PCA and also prevents immediate hypersensitivity reactions in rats and monkeys. It also inhibits the release of slow releasing substance of anaphylaxis (SRSA)^{ovv}.[72] In a series of 2-nitroindan-1,3-diones, 22 on subcutaneous administration is 39 times DSCG in rat PCA and is also orally effective.[73] By contrast, hydroaromatic analogs lose activity.[74]



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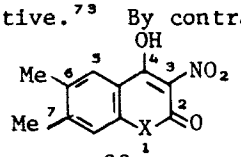
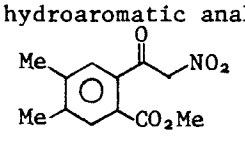


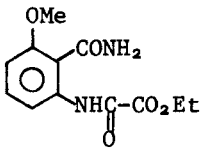
23



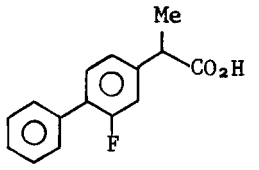
24

The closely related 23 is less active in rat PCA (6.7 times DSCG), and structure-activity relationships for this class of compound have been reported. When X=NR, activity is maintained when R=Me but decreases rapidly when R=longer alkyl. Alkyl groups at positions 6 and 7 are optimal. Replacement of the benzene ring by pyridyl maintains activity but only when the N is at position 7. Reduction of the phenyl ring (hydroaromatic) decreases activity.[75] Analogs of 22 in which the di-keto nitro ring has been cleaved to an ω-nitroacetophenone derivative 24 are also active in rat PCA. However, in vivo, cyclization to 22 is a prerequisite for activity and the conversion of 24 to 22 is greater than 90% within 2 hrs. in rats.[73]

Of a series of N-aryl and N-heteroaryl oxamic acid esters 25 is active in rat PCA both orally and intravenously. Oral activity is lost by the conversion of CO_2Et to CO_2H.[76]



25



26

Flurbiprofen (26) antagonized the effect of SRS-A on the tracheal chain but not the action of SRS-A on guinea-pig ileum, suggesting that the receptor sites for SRS-A differ on different organs.[77] Pyridoxine therapy (200 mg daily) showed significant improvements in childhood asthma and a reduction of the therapeutic dosages of bronchodilators and cortisone required. These children appear to have a deficiency in tryptophane metabolism.

Peptides - A synthetic peptide Asp-Ser-Asp-Pro-Arg has been proposed to be identical with an amino acid sequence in IgE, and has been claimed to be effective in blocking the allergic response to guinea pig dander in 6 patients.[79]

Emphysema - This is chronic respiratory disease of unknown etiology in which alterations of the lung leads to progressive alveolar wall destruction. Major α-antitrypsin (α-AT) deficiencies are associated with a predisposition to emphysema, by a mechanism believed to involve proteolytic enzymes derived from leukocytes and macrophages.[80] α-AT deficiency represents less than 5% of the total emphysema population.[81] Severe and intermediate α-AT deficiencies may result from homozygous and heterozygous inheritance of an abnormal gene respectively. Although the homozygous state is strongly predisposed to the development of pulmonary edema even an intermediate deficiency may contribute to disease development.[82, 83] A variety of systems have been used to induce experimental emphysema including intratracheal instillation of enzymes, e.g. elastase, which produces lesions resembling panacinar emphysema.[84] Papain-induced emphysema increased the porosity of the pulmonary epithelium since lipid insoluble drugs, e.g. mannitol, p-aminohippuric acid, were absorbed 2 times as rapidly after tracheal administration in papain treated rats than in controls.[85] Emphysema induced in hamsters by intratracheal injection of 25 units of porcine pancreatic elastase was morphologically similar to human congenital lobar and panlobar emphysema. A sim-

ilarity between human panacinar emphysema and that induced by hamsters by elastase was noted by scanning electron microscopy.[86] These observations are consistent with the hypothesis that the emphysema of α-AT deficiency is related to inadequately inhibited proteolysis.[86] The pregnenes, progesterone and medroxyprogesterone acetate but not norethindrone (19- nortestosterone derivative), partially prevented the papain induced breakdown of alveolar septa in rats. Paramethasone and indomethacin were ineffective, and only limited protection was afforded by cyclophosphamide and the proteolytic enzyme inhibitor, aprotinin.[87] An extensive review of other mechanisms concerning the pathogenesis of emphysema including developmental and anatomical aspects has also appeared.[88]

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Chapter 7. Antihypertensive Agents

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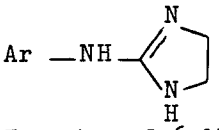
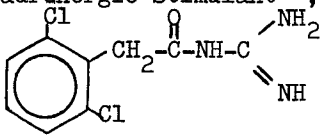
This review covers recent advances in the study of antihypertensive agents including β-adrenergic blockers and antihypertensive vasodilators but excluding diuretics. Several substantial reviews have appeared on the development and classification of hypertension[1,2,3] and its drug treatment[4,5,6,7] Adverse reactions and interactions limiting the use of antihypertensive drugs have been collated[8].

Centrally Acting Antihypertensive Agents - Cardiovascular regulation by central adrenergic mechanisms has continued to attract attention[9,10,11] Two brain stem systems have been proposed for the control of blood pressure[12,13] and the role of serotonin[14] and histamine[15] in central cardiovascular control have been investigated. Clonidine (Boehringer-Ingelheim), Ia, the centrally acting alpha-adrenergic stimulant[16], has been



Ia Ar = 2,6-dichlorophenyl
Ib Ar = 2 Me, 5-fluorophenyl



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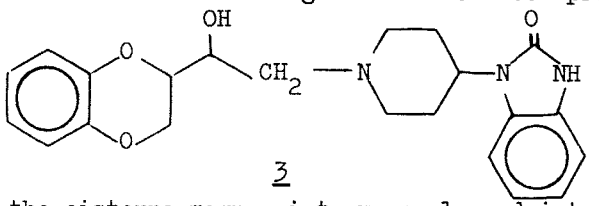
studied further. In rat experiments it is reported to inhibit both noradrenergic and serotoninergic neurons in the brain[17,18], and its positive inotropic effect on the isolated guinea pig heart indicates an H_2-histaminergic effect[19]. Clonidine causes a decrease of plasma and urinary catecholamines, plasma renin activity and urinary aldosterone in hypertensive patients[20]. In studies in dogs, the reduction in plasma renin activity was identified as being the result of a centrally mediated reduction in renal sympathetic neural tone[21], although in the isolated rat kidney a direct renin reducing effect was found[22]. In man, the fall in blood pressure with clonidine is not correlated with the change in plasma renin activity[23]. In the anaesthetized dog clonidine suppresses antidiuretic hormone secretion[24] Experiments in rabbits suggest that it causes a dose dependent increase in the gain of the baroreflex with an action in the brain and at the baroreceptor[25,26] Combipres ®, a combination of clonidine and chlorthalidone, has been introduced into the U.S.A. (Boehringer-Ingelheim)[27a] It has been reported to be suitable for long term treatment[28], ST600 (Boehringer-Ingelheim) Ib has been compared with clonidine in man[29]. At equihypotensive doses they have similar cardiorenal hemodynamic effects but ST600 is reported to be longer acting and to produce milder side effects of dry mouth, sedation and constipation. The structure activity relationships in a series of imidazoline ∝-stimulants have been reported[30,31,32] Peripheral alpha-adrenergic activity is correlated primarily with pK_a. The bradycardia produced in rats by intrahypothalamic injection suggests that structural requirements for central alpha-adrenergic activity differ from those for the periphery. This is

not just due to the differences in the ability of the drugs to reach the receptor; there are differences in the drug receptor interactions. Molecular geometry and adrenergic drug activity have been reviewed[33].

The chemistry[34] and pharmacology[35,36] of BS 100-141 (Sandoz) (2) have been reported. It is a central and peripheral alpha-adrenergic stimulant which resembles clonidine in many respects but with important differences. Unlike clonidine it is ineffective in lowering the blood pressure in the cat following direct application to the ventral surface of the medulla oblongata. This suggests a different, though as yet unknown, site of action within the CNS. BS 100-141, unlike clonidine, does not inhibit dopamine turnover in the corpus striatum of the rat. It is very much less sedative than clonidine as judged by observation in dogs and by rat EEG studies[37]. Initial studies in man indicate that it produces fewer and less severe side effects than clonidine[38].

A novel centrally acting hypotensive agent has been described[39-43], Janssen R28935 (3). In anaesthetized dogs it lowers blood pressure when



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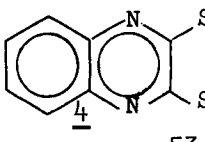
administered into the cisterna magna, intravenously and into the vertebral artery. The blood pressure lowering effect is not inhibited by piperoxan, desmethylimipramine or nalorphine. Unlike clonidine it does not cause vasoconstriction and in fact causes a decrease in total peripheral resistance. The compound is not a central or peripheral alpha-adrenergic stimulant and at hypotensive doses no systemic α- or β-adrenergic blocking effect could be found. The erythro form of R29835 is much more effective than the threo. In renal hypertensive beagles at 1.25 mg/kg p.o. it gave a marked and long lasting fall in blood pressure without significant bradycardia.

Indoramin[16] (Wyeth 21901) has been shown to have both central and peripheral antihypertensive actions. In anaesthetized cats 1 mg/kg i.v. lowered blood pressure without a significant influence on efferent sympathetic nerve activity but 5 mg/kg i.v. lowered blood pressure further and inhibited activity in the splanchnic, cardiac and renal nerves[44].

The Renin-Angiotensin System - (The effects of β-blockers on this system, are mentioned in a later section). Attempts have been made to classify essential hypertension into sub-groups on the basis of plasma renin levels[45-47]. The role of the system in the pathogenesis of various types of hypertension has been reviewed[48] and its relationship to aldosterone metabolism[49] and the sympathetic nervous system has been mentioned[50]. The dithiol 4 (Squibb) is an angiotensin I (AI) converting enzyme inhibitor. It decreased the pressor response to AI but only slightly to angiotensin II (AII) in rats[51]. It is now believed[52] that AII is further converted to



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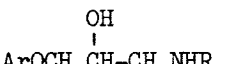
angiotensin III (AIII), the heptapeptide des-Asp^{1}-AII. AII is the vasoconstrictor substance and AIII mediates aldosterone release. Both agents have an effect on renin release[53] which may be consistent with the hypothesis that the heptapeptide mediates the negative feedback loop.[53,54] Des Asp^{1}-Ile^{8}-AII inhibits aldosterone secretion but not the AII pressor response in rats and dogs whereas Sar^{1}-Ile^{8}-AII inhibits the pressor response of AII but not aldosterone secretion.[55,56] The effect of several AII agonists and antagonists on steroidogenesis in isolated dog glomerulosa cells has been investigated and structural elements leading to partial agonist activity have been identified[57]. The most widely studied AII antagonist in animals is saralasin, Sar^{1}-Ala^{8}-AII (Eaton Labs, P-113, 'Sarenin')[16] and recent studies of saralasin in man have made a significant contribution to the understanding of various types of hypertension. An "angiotensinogenic" hypertension has been described[58]. Out of 221 consecutive patients 32 responded to saralasin. Many of these patients also showed evidence of renal ischaemia. Indomethacin has been shown in man to reduce the furosemide induced increase in plasma renin levels in parallel with inhibition of renal prostaglandin synthesis[59].

Beta-adrenergic blocking agents (β-blockers) - The antihypertensive properties of β-blockers were discussed last year[60]. Their medicinal chemistry, mode of action[61,62] and clinical use[63] have been comprehensively reviewed. The mode of action in hypertension of these drugs is still unclear but some new evidence has appeared. It is now generally accepted that reduction in cardiac output does not explain their hypotensive action.[61,62,64] The original suggestion that the depressor effect of β-blockers was correlated with the fall in plasma renin levels[65] has not been confirmed[46,66,67,68] but in the light of recent work with saralasin it has been suggested that such a correlation might be expected only for patients with "angiotensinogenic" hypertension and adrenergically mediated renin release[58]. In so far as renin levels reflect the level of sympathetic nerve activity in essential hypertension, measurement of renin levels may be useful in predicting the patients response to β-blockers[69]. Studies with agonists[70] and antagonists[69] indicate that renin release is mediated by β_1-receptors. With propranolol (5) (ICI), which has been used clinically for 8 years in hypertension in Europe, the fall seen in plasma renin occurs more rapidly than the fall in blood pressure[71] and at much lower doses[72] leading to the suggestion that β-blockers have a low dose effect associated with renin suppression and a high dose effect which is renin independent[73]. The central action of β-blockers is believed to cause a reduction in sympathetic nervous tone[9,74]. Attempts have been made to correlate the effects of β-blockers with their absorption and distribution within the brain[75]. A relaxation of the vascular bed and decrease of blood pressure may occur simply as a result of the blockade of surges in cardiac output and blood pressure produced by stress[61,62].

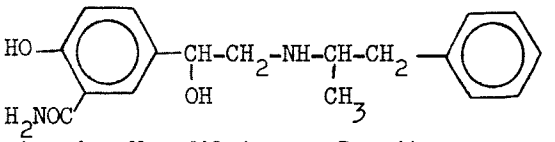
Acebutolol ('Sectral') (6) has been launched by May and Baker in Britain[27b]. It is a cardioselective agent with some intrinsic stimulant

activity[76]. Metoprolol (7) has been launched in Britain by Hässle ('Betaloc') and by Ciba-Geigy ('Lopressor')[27c]. It is a potent cardioselective agent with no intrinsic stimulant activity[77,78]. Timolol (8) launched last year by Merck in Britain[60] has been reviewed[79] Penbutolol (Hoechst 893d) (9) a non selective agent[80] and atenolol ('Tenormin', ICI 66,082) (10) a cardioselective agent with neither partial agonist activity nor membrane stabilising activity have both been found to be effective in man[81,82]. Correlation between the response of hypertensive patients to β-blockers and numerous physiological parameters has been sought but not found to date[82]. On the basis of evidence accumulated so far the best profile of activity for an antihypertensive β-blocker is long duration and cardioselectivity without intrinsic sympathomimetic activity[61]. The complementary action of β-blockers (in controlling tachycardia and renin release) and vasodilators has been reviewed[83] and the renin suppressing activities of β-blockers in acute and chronic diuretic treatment have been discussed.[84]



		Ar	R
5	Propranolol	1-naphthyl	iPr
6	Acebutolol	4-butyramido 2-acetylphenyl	iPr
7	Metoprolol	4-MeO-CH_2CH_2-phenyl	iPr
8	Timolol	3-(4-morpholino) 1,2,5-thiadiazolyl	tBu
9	Penbutolol	2-cyclopentylphenyl	tBu
10	Atenolol	4-H_2NCOCH_2-phenyl	iPr

The α and β-blocker labetalol (Allen and Hanburys 5158A) (11) has been reported to lower blood pressure in hypertensive patients with an effect similar to the combination of propranolol and hydralazine. Postural hypotension was observed only at higher doses[85].



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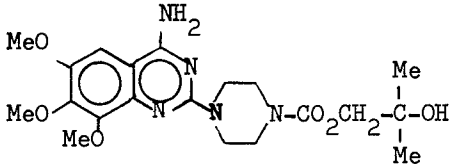
Antihypertensive Vasodilators - Despite numerous reports of peripheral vasodilators over the past few years there are very few agents sufficiently potent and long acting to be useful in the treatment of hypertension. The use of vasodilators in hypertension and the control of the attendant side effects of tachycardia and fluid retention has been reviewed.[83,86,87] Prazosin[16] (Pfizer) has been used alone[88] but an increased antihypertensive effect is observed when it is combined with a diuretic[89] or propranolol[90]. In some patients side effects of collapse with hypotension and marked tachycardia have been observed with prazosin.[91] The analogue trimazosin(12)has had a limited clinical trial.[92] It does not appear to cause postural hypotension or tachycardia at doses between 150 and 500 mg/day and does not have alpha-adrenergic blocking properties. The metabolism of minoxidil[16] (Upjohn) has been studied in dogs, rats,

monkeys and man[93]. Man and monkeys exhibited a similar metabolite profile with the O-glucuronide as the major metabolite.

P-RG-138-Cl (Pharma-Research) (13) has been evaluated in man[94]. It lowers mean arterial pressure at doses of 3-10 mg/kg p.o. Patients suffered tachycardia and there was a high incidence of headache and postural dizziness but no sign of fluid retention.



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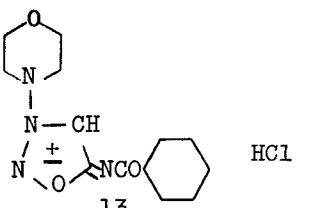

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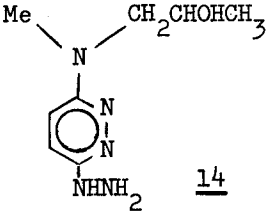
The medicinal chemistry of ISF 2123 (14) has been described[95] and the first clinical reports have appeared[96]. In renal hypertensive rats it is ten times more potent than hydralazine in lowering blood pressure and is six times less toxic (mouse LD50). In man, at doses of 6 mg/kg/day p.o. it normalised blood pressure without marked tachycardia.



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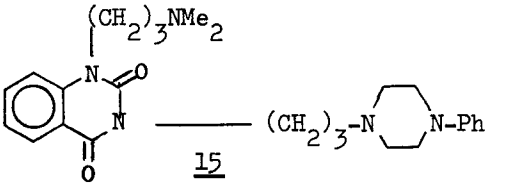

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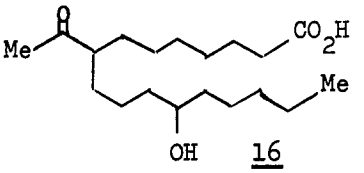
N-alanyl dopamine (Abbott 37301) a renal vasodilator, when given orally to hypertensive patients caused a significant increase in renal plasma flow but did not modify blood pressure[97]. The quinazoline (15) (Miles Labs) is reported to be a vasodilator in the dog femoral bed and to lower blood pressure for up to 8 hrs in renal hypertensive rats without significant alpha-adrenergic blockade[98]. The novel secoprostaglandin (HHDA, Merck) (16) has been described as an antihypertensive vasodilator. It increases renal blood flow in the dog and lowers the blood pressure of spontaneously hypertensive rats at 10-20 mg/kg[99].



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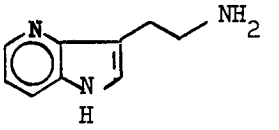

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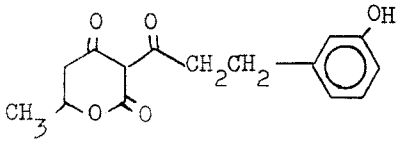
Miscellaneous mechanisms and agents - Serotonin may play a peripheral as well as central[14] role in the maintenance of vasomotor tone. Chronic oral administration of p-chlorophenylalanine, a serotonin synthesis inhibitor, lowers blood pressure in genetically hypertensive rats[100]. The azatryptamine 17 reduced blood pressure in cats at doses of 0.1 to 0.25 mg/kg[101]. The availability of selective H_1 and H_2-histaminergic agonists

and antagonists has allowed further study of the role of histamine in the control of blood pressure[102]. The role of cyclic nucleotides[103] and prostaglandins[104] in the biochemical etiology of hypertension have been reviewed. It has been suggested that a deficiency in prostaglandin dehydrogenase may be the primary genetic lesion in genetically hypertensive rats[105].

Pyratrione (Toray) (18), a tyrosine hydroxylase inhibitor, has now been tested in man. It reduced blood pressure in 28 of 39 patients when



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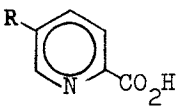

19a R = 4 HO-C_6H_4-CH_2
19b R = Bu
19c R = 4-Cl-Bu

dosed at 300 to 900 mg/day for 3 weeks[106]. Phenopicolinic acid (19a), a microbial product, is twice as potent as fusaric acid (19b) as a dopamine-β-hydroxylase inhibitor and lowered blood pressure in spontaneously hypertensive rats by 20% 5 hours after dosing at 50 mg/kg p.o.[107]. PD-008 (19c) (Nippon Kayaku) is also more potent than fusaric acid. It lowers blood pressure in rats for up to 24 hours at doses of 12.5 mg/kg[108]. The use of β-homoaminoacids has allowed the preparation of [7-β-H Pro] bradykinin which is equipotent with bradykinin as a depressor agent but of longer duration[109].

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Chapter 8. Diuretics

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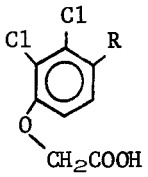
The advent of the thiazide diuretics was followed by the discovery of the loop diuretics and then the antikaliuretic agents. A decade or more passed with no new plateaus of advancement in this field. Within the recent past, several noteworthy trends in diuretic development have emerged. Uricosuric saluretic agents which are more potent than the mercurials have been discovered. Greater structural novelty and diversity are observed with a higher proportion of compounds that are organic bases, many of which are reported to exhibit minimal kaliuretic activity. Finally, a much clearer picture is evolving concerning the role and nature of such renal mediators as the prostaglandin (PG) and kallikrein-kinin systems.

Aryloxyacetic Acid Diuretics - The effect of ethacrynic acid on renal blood flow (RBF) has been examined. Eide et al[1] infused ethacrynic acid i.v. into anesthetized dogs after inhibiting sympathetic mechanisms of renin release; RBF rate increased by 54% and renin release increased from 0.8 to 16.4 μg/min. During arterial constriction, ethacrynic acid had no additional effect on renovascular resistance or on renin release. It was proposed that ethacrynic acid increases renin release through a hemodynamic mechanism triggered by afferent arteriolar dilation, and inhibits renin release by increasing the delivery of $Na^{\oplus}$ to the distal convoluted tubules. In order to test the possibility that ethacrynic acid might be releasing vasodilating PG's, indomethacin, an inhibitor of PG synthesis, was employed. Renal arterial administration of ethacrynic acid to dogs greatly increased RBF, whereas a second dose after indomethacin administration produced only a slight increase in RBF. This greatly diminished increase in RBF after indomethacin administration implicates prostaglandins as mediators of the renal vasodilation produced by ethacrynic acid.[2]

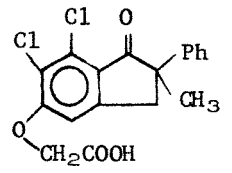
Cragoe and co-workers[3-6] have reported four series of diuretic substituted-vinylaryloxyacetic acids. The two most active series were the diacylvinyl- and nitroalkenylphenoxyacetic acids, illustrated by compounds



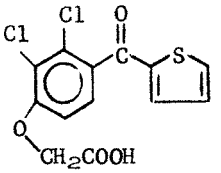
R	No.	R	No.
$C_2H_5C(=CH_2)CO-$	Ethacrynic Acid	$CH_3COCH=CH-$	3
$(CH_3CO)_2C=CH-$	1	CNCH=CH-	4
$CH_3(NO_2)C=CH-$	2		

1 and 2, which were qualitatively similar in action to ethacrynic acid in inducing prompt diuresis upon oral administration to dogs and in inducing the excretion of $Na^{\oplus}$ and $Cl^{\ominus}$ in approximately equimolar quantities. However, these compounds were 3-5 times as potent as ethacrynic acid. The monoacylvinyl analogs were less active, e.g., 3 possesses only half the potency of ethacrynic acid. Similarly, other substituted vinylphenoxyacetic acids were also less potent, as illustrated by 4 which was about 1/10 as potent as ethacrynic acid.

Evaluation of the uricosuric diuretic MK-196 in chimpanzees has been reported.[7,8] At an oral dose of 2.5 mg/kg, uric acid excretion (C_{urate}/GFR) is increased five-fold by this compound. Intravenous doses of 1 mg/kg rapidly increase C_{urate}/GFR from a control value of 0.10 to 0.70 which is maintained throughout the observation period. Urine flow rates as great as those induced by ethacrynic acid or furosemide were obtained after 30 minutes when Na^+ excretion is maximal (2300 μEq/min). The uricosuria induced by MK-196 was not reversed by pyrazinoic acid as was observed with probenecid. Elucidation of the major metabolites, primarily the 2-(p-hydroxyphenyl) derivative, of MK-196 has been reported.[9,10]



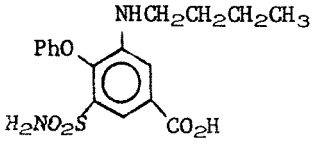
MK-196



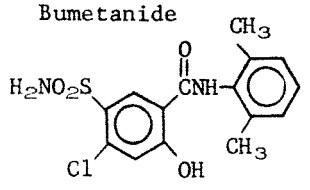
Ticrynafen

The synthesis and pharmacology of ticrynafen (tienilic acid) and some of its analogs where the 2-thienyl moiety is replaced by other heterocyclic radicals has been published.[11] Mouse data indicated no compound to be more active than ticrynafen except the 2-furyl analog which was more diuretic but less uricosuric. A double blind randomized crossover study[12] comparing ticrynafen (at both 250 mg/day and 125 mg/day) with probenecid (500 mg/day) in six hyperuricemic patients showed that serum uric acid reduction was greater with ticrynafen at either dose than with probenecid. There was an increase in urate clearance with both doses of ticrynafen, but neither was significantly greater than that produced by probenecid.

Sulfonamide Diuretics - A two-day symposium on bumetanide was held at the Royal Society of Medicine, London.[13] The twenty papers presented involved reports on the structure-activity relationships, pharmacology, pharmacodynamics and clinical efficacy of bumetanide. Evidence for the metabolism of radiolabeled bumetanide has been presented[14] indicating that 81% of the dose was excreted in the urine and the remainder in the feces within 48 hours. Although 63.5% of urinary ^{14}C was unchanged bumetanide, the metabolites identified indicate metabolism occurs on the butyl side chain, with the primary alcohol as the major metabolite.



Bumetanide



Xipamide

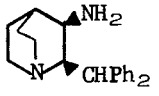
A clinical crossover study comparing xipamide with furosemide concluded that the two diuretics were equipotent, but that xipamide has a longer duration of natriuresis (12 hours) than does furosemide (4 hours).[15] A structure-activity study for xipamide and a number of its congeners indicated that all changes in the molecular structure of xipamide including acetylations, introduction of a second sulfamoyl moiety or replacement of the carbonyl group by a sulfonyl group led to decreased activity.[16]

Hypokalemia continues to be a major undesirable side effect associated with long-term diuretic therapy with

sulfonamide diuretics. A number of recent papers describe the clinical efficacy of the combination of hydrochlorothiazide (50 mg) and amiloride (5 mg) (MODURETIC®) in normal,[17] edematous[18,19] and hypertensive[20] patients in averting the problem of hypokalemia. In general, marked diuresis was observed in all cases. Upon treatment of digitalized edematous patients with MODURETIC®, urine volume doubled and peaked at 2-4 days. Urinary $Na^{\oplus}$ increased and urinary $K^{\oplus}$ decreased; no significant change was observed in serum electrolyte concentrations.

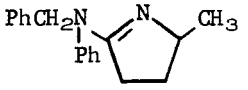
In the long-term treatment of 47 hypertensive patients,[20] the hypotensive response to MODURETIC® was comparable to that obtained with other diuretics; however, the serum $K^{\oplus}$ levels remained normal during the course of 12 months.

Diuretic Bases - A number of 3-amino-2-benzhydrylquinuclidines have been synthesized and tested for diuretic activity in both rats and dogs.[21] The most active member studied was cis-3-amino-2-benzhydrylquinuclidine (5) which in dose-response studies in rats had a ceiling oral saluretic effect greater than hydroflumethiazide but less than furosemide. Similar effects were seen in oral studies in dogs; however, 5 displayed an unusual biphasic dose-response saluretic effect with the peak activity appearing at about 10 mg/kg. In both species the $Na^{\oplus}/K^{\oplus}$ ratios were about the same as the two standard drugs. The trans isomer of 5 as well as derivatives of 5 bearing substituents on the amino group or on the aromatic nuclei were less active than 5.



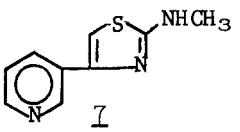
5

A series of N-phenylamidines with diuretic activity have been described.[22] For example, 5-methyl-(2-N-phenylbenzylamino)-1-pyrroline (6) in the Lipschitz rat assay when administered orally at 6.25 mg/kg, when compared to the urea control group, afforded a ratio of 2.32 for volume excretion, 2.03 for $Na^{\oplus}$ and 0.75 for $K^{\oplus}$ excretion. The $Na^{\oplus}/K^{\oplus}$ ratio for 6 is 2.7, whereas the value for hydrochlorothiazide under the same conditions is 1.65.



6

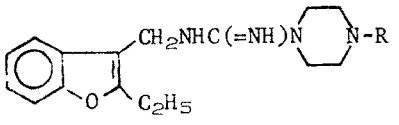
Data supporting a claim of an improved $Na^{\oplus}/K^{\oplus}$ ratio was presented for a series of 2-amino-4-pyridylthiazoles.[23] Using the Lipschitz rat assay in which the drugs were administered orally, the following data for the most active compound (7) were obtained in 5 hours in comparison with chlorothiazide. Analogs of 7 bearing other N-substituents, isomeric pyridyl substituents at the 4-position or 5-alkyl substituents were less active than 7.



7

Compd.	Dose mg/kg	Vol. (ml/kg)	$Na^{\oplus}$	$K^{\oplus}$	$Cl^{\ominus}$	$Na^{\oplus}/K^{\oplus}$
7	50	12.1	3.12	1.93	4.07	1.61
	5	20.1	2.25	1.55	2.70	1.45
Chloro-thiazide	6	7.64	1.72	1.19	1.97	.68

Both diuretic and hypotensive activity has been reported[24] for a series of 2-(2-alkyl-3-benzofuranyl)-N,N-(3-alkyl-3-azapentamethylene)acetamidines of type 8. Some of the more active members of the series are listed in the following table. The hypotensive effects were measured in the Grollman[25] renal hypertensive rats; normal rats were used for diuretic evaluation. The drug dose for each assay was 100 mg/kg, p.o.

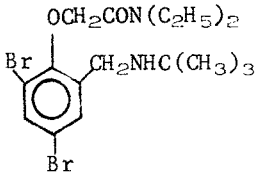


8

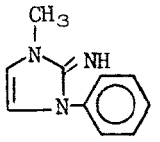
Compd. 8 R=	mm Hg Reduction in B.P.	% Increase in Urinary Excretion
$-CH_3$	25	130
$-C_2H_5$	35	-
$-(CH_2)_2CH_3$	25	70
$-CH(CH_2)_2$	15	130

When the 2-alkyl substituent was other than ethyl, less active compounds resulted. However, activity was retained when R was H, lower alkyl or ethoxycarbonyl. In addition, compounds where RN was replaced by methylene retained activity.

In a 6 hour oral dose-response study in rats, Se 852·Hydrochloride was compared to furosemide at doses of 10, 20, 40 and 60 mg/kg. Se 852·Hydrochloride was more diuretic at all doses, and it was equisaluretic at the two lower doses.[26] However, at the two higher doses, furosemide was more saluretic and less kaliuretic.



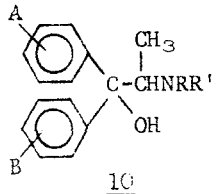
Se 852



9

Azolimine (9) has been shown to produce natriuresis in adrenalectomized rats in the absence of exogenous mineralcorticoids; its effectiveness was greater in the presence of a steroid agonist.[27] In conscious dogs given an infusion of saline and dextrose, azolimine was only effective when deoxycorticosterone was administered. This compound appears not to be a pure competitive antagonist of mineralocorticoid, but its greater efficiency in the presence of mineralocorticoid distinguishes it from both the pure competitive antagonists (i.e., spironolactone) and the non-competitive antagonists (i.e., amiloride). Azolimine significantly improved the urinary $Na^{\oplus}/K^{\oplus}$ ratio when used in combination with hydrochlorothiazide, quinethazone, or furosemide.

A series of 1,1-diaryl-2-disubstituted-aminopropanols (10) has been disclosed[28] which exhibited saluresis with little kaliuresis above control values.

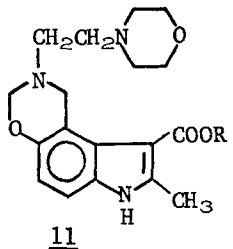


10

No.	A	B	NRR'
T-2259	2-OMe-5-t-Bu	H	$N(CH_2)_5$
T-2455	2-OMe-3-Me-5-t-Bu	2,4-$(OMe)_2$	$N(CH_2)_5$
T-2464	2-OMe-3-Me-5-t-Bu	3,4-$(OMe)_2$	$N(CH_2)_5$
T-2493	3,5-Me_2-4-OMe	2-OMe-3-Me-5-Cl	NMe-t-Bu

The test compounds were evaluated orally in rats at approximately 15 and 30 mg/kg in comparison to triamterene at 10 and 20 mg/kg and hydrochlorothiazide at 3 mg/kg. Under these conditions, the test compounds were more diuretic and saluretic than hydrochlorothiazide or triamterene. They were considerably less kaliuretic than hydrochlorothiazide but not as antikaliuretic as triamterene. For example, at the higher dose the $Na^{\oplus}/K^{\oplus}$ ratio for triamterene was 13.2 in comparison to 12.9 and 10.7 for T-2455 and T-2464, while the latter two were producing a much greater diuresis and saluresis than triamterene. Interestingly, these compounds possess a very low order of acute oral toxicity in mice in comparison to triamterene.

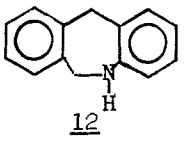
Oral studies[29] in saline loaded mice have revealed some compounds of the series illustrated by 11 to be more diuretic than furosemide. For example, the dose of furosemide required to increase the urinary volume to double that of controls within three hours was 12.5 mg/kg. The value for the three analogs of 11 was 4.3, 8 and 9, respectively. The corresponding value for 72365 in rats was 0.5. The acute oral toxicity of the three compounds in rats was quite low.



11

No.	R
72365	Et
72730	Pr
72762	Me

Using non-fasted, water loaded rats, 12 has been evaluated orally for its saluretic and diuretic effects at 25, 50 and 100 mg/kg and found to produce a 2.8, 4.2 and 5.0 fold increase in sodium excretion, respectively, while $K^{\oplus}$ excretion increased by only 1.9 fold at each dose level.[30]



12

Prostaglandins - Several excellent reviews[31-34] examining the possible role(s) played by the renal prostaglandins (PGE_2, PGA_2 and $PGF_2\alpha$) in renal function were published during the past year. Recent investigations have been directed primarily toward the elucidation of the sites and modes of metabolism and the intrarenal transport of renal PG's. Also, the role(s) of PG's has been clarified in regard to their interrelationships (at endogenous levels) with other regulators of kidney function, i.e., renin-angiotensin-aldosterone, kallikrein-kinin, vasopressin and erythropoietin, as well as with various diuretic agents. These studies have proved to be both fruitful and provocative and constitute the subject of this brief review.

Frölich and colleagues[35] recently obtained definitive evidence (using mass spectrometry) needed to confirm their preliminary observation[36,37] that low levels of PGE_2 and $PGF_2\alpha$ are present in human (ambulatory female volunteers) and animal (mongrel dogs) urine. More importantly, their current studies demonstrated that urinary PG's may originate from the kidney, thus indicating that renally synthesized PG's either diffuse into or are secreted directly into the tubule. Furthermore, these workers have suggested that urinary PG's are a reflection of renal PG synthesis and, thus, may constitute a potentially valuable tool for delineating renal PG physiology and pathology.

The above observations led Frölich et al[35] to hypothesize that medullocortical PG transport occurs via the tubular fluids to the site where the tubule is in direct apposition to glomerular arterioles. Interestingly, this hypothetical intrarenal transport pathway is critical to the rationale of a recent hypothesis by Oken[38] implicating the role of PG's in the pathogenesis of acute renal failure (ARF). According to this hypothesis, renal PG's synthesized in the medulla, enter the loop of Henle and travel to the macula densa via the tubular urine, thus escaping otherwise likely deactivation by 15-hydroxy PG dehydrogenase (15-HPGDH) in the cortex.[39] Generally, under most conditions of diminished GFR and renal perfusion, flow through Henle's loop is maintained, albeit at a reduced rate, and renal PG release is augmented.[40] These combined factors could lead to a high concentration of vasodilatory PG's reaching the macula densa and thereby effectively counteracting vasoconstrictor stimuli. However, if filtration completely ceases due to insurmountable vasoconstriction, flow along the loop would stop and PG's could no longer reach the macula densa. Thus, the PG feedback loop would be severed, vasoconstriction would be unchecked and filtration failure perpetuated. Hence, Oken suggests that impaired glomerular filtration (observed) in ARF may be due, if only in part, to the derangement of a normal feedback mechanism involving PG's.

Recent studies of Korobkin and co-workers[41,42] in dogs with norepinephrine-induced ARF have shown that infusion of PGE_1 (1.0 μg/min in 0.45 N saline) into the renal artery at the rate of 2.25 ml/min for 1 hr promptly increased urine volume from 3.6 $\pm$ 0.8 to 15.2 $\pm$ 5.4 ml/45 min, produced a 12-fold increase in $Na^{\oplus}$ excretion from 0.14 $\pm$ 0.05 to 1.65 $\pm$ 0.77 mEq/45 min and increased total RBF to the infused kidney by 114%. Diuresis was not accompanied by a significant rise in the endogenous creatinine clearance; cardiac output also remained unchanged. The rate of tissue perfusion to each of the three cortical zones was significantly increased; in addition, intrarenal flow was redistributed from the outer to the juxtamedullary cortical zone. The authors pointed out that the dissociation of RBF from creatinine clearance is consistent with the view that glomerular perfusion was augmented by a mechanism that did not permit augmentation of the GFR.

Levine and colleagues[43,44] have continued to investigate the distribution and properties of both 9-keto PG reductase (9-KPGR) and Types I ($NAD^{\oplus}$-dependent) and II ($NADP^{\oplus}$-dependent) 15-HPGDH in the renal medulla and cortex of various mammalian species. In swine kidney, 9-KPGR was equally distributed in the medulla and cortex. Eleven times more Type I 15-HPGDH was present in the cortex than in the medulla, whereas, the medulla was shown to contain twice as much Type II 15-HPGDH as the cortex. It has been observed that PGE_2 is the most abundant medullary PG and, additionally, is a relatively poor substrate for Type II 15-HPGDH, the major medullary dehydrogenase. This led Levine et al[43] to suggest that the coupled NADPH-dependent conversion of PGE_2 to $PGF_{2\alpha}$ and the $NADP^{\oplus}$-dependent inactivation of the product, $PGF_{2\alpha}$, might serve to regulate the extent and duration of PGE_2-induced vasodilation in the renal medulla as first observed by McGiff.[45] Stone and Hart[46] recently reported the isolation and characterization of a 9-KPGR from rabbit renal cortex and, surprisingly, found that

PGA_2 was not a substrate for their 9-ketoreductase. The significance of this observation awaits future elucidation of the genesis of PGA_2 which presently remains unresolved.

In an elegant series of recently published papers, McGiff and others [47-53] presented an impressive mass of experimental data focusing on the possibility that the renal kallikrein-kinin system may be a prime regulator of kidney function. Although there appears to be sharp disagreement amongst these workers as to the importance of this system in regulating salt and water excretion, they seem to agree that (a) kinins may be prominent mediators of RBF, (b) an important relationship may exist between secretion of mineralcorticoids and excretion of kallikrein and (c) the distinct possibility exists that renal kallikrein deficiency may contribute to the genesis of hypertension. An interaction between the renal PG's and kinins was first suggested by McGiff and colleagues[54] based on the observation that intrarenal arterial infusion of bradykinin, but not of eledoisin (another vasodilatory peptide), increased the release of PGE-like compounds into the renal venous blood of the canine kidney. Numerous subsequent studies by McGiff and others have led McGiff et al[48] to propose that the coupling of the kallikrein-kinin and PG systems within the kidney may be unique. They point out that PG's mediate some of the actions of kinins and modulate others, while depending on the intrarenal generation of kinin to set up their level and type of activity. Thus, according to these authors, not only production of PG's but the functional consequences within the kidney of their enhanced production, as determined by the ratio of PGE_2 to $PGF_{2\alpha}$, may be subject to regulation by kinins originating intrarenally.

The well-known increases in RBF and saluresis, as well as the hypotensive effects, elicited by the loop diuretics, furosemide and ethacrynic acid, may be mediated (at least in part) by renal PG's.[2,55] To explore the possible relationship between these diuretics and renal PG's, Lee et al[56] investigated the effects of furosemide and indomethacin, a potent PG biosynthesis inhibitor, administered individually and in combination in a series of four normotensive and six essential hypertensive patients on a restricted salt diet. According to this study, indomethacin substantially diminishes the clinical effects of furosemide including natriuresis and reduction of elevated blood pressure. Hence, these studies support the possibility that renal PG's may mediate the clinical effects of furosemide.

In conclusion, design and evaluation of future diuretics will no doubt be greatly influenced and tempered by an acute awareness of the physiological role(s) played by the renal PG's in kidney function; the full story is yet to emerge.

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Chapter 9

Prostaglandin Structure-Activity Relationships

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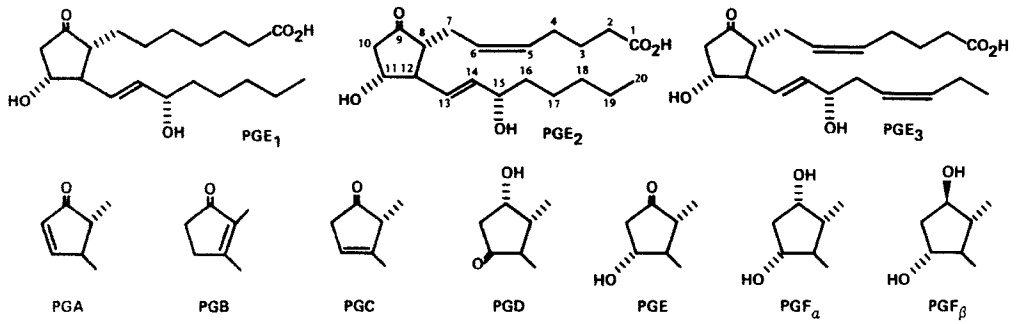
Although over 45 years have passed since the detection of prostaglandin activity[1] and almost 15 years since the first prostaglandin structure was published[2], the precise physiological role and mechanism of action of this family of extremely potent C_{20} fatty acids (Chart I) remain to be unequivocally elucidated. Extensive pharmacological evaluation of these unique natural products has, however, uncovered a broad spectrum of activities, both in animals and man, which has been described both in excellent general reviews[3-5] and specific reviews dealing with recent developments in synthesis[6], metabolism[7], antagonists[8], reproductive physiology[9], cyclic nucleotides[10], hematology[11], pulmonary aspects[12], gastrointestinal effects[13] and cardiovascular activity[14].

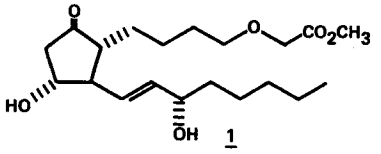
CHART I



Despite the demonstration of potent pharmacological activity, the original optimistic predictions of clinical utility for the natural prostaglandins have not been realized. The lack of selectivity and oral efficacy, chemical instability and short duration of action of the natural materials have limited their commercial use to prostaglandin $F_{2\alpha}$ (dinoprost tromethamine)[15] as an abortifacient and prostaglandin E_2 (dinoprostone)[15] both as an abortifacient and inducer of labor. However, from the perspective of the medicinal chemist the prostaglandins offer a great opportunity for the development of useful therapeutic agents, and many laboratories have reported the preparation of a wide array of analogs over the past decade. Unfortunately, many of these reports do not provide the biological data necessary to evaluate the effect of structural change on activity. In addition, conclusions concerning prostaglandin analog activity must be accepted with caution unless data for several biological assays is presented, since inactivity in one assay may be merely a reflection of selectivity. Nevertheless, in the discussion below a number of useful prostaglandin structure-activity relationships (SAR), derived over the past decade, has been summarized[16].

Carboxylic Acid Side-Chain: The finding by Kloeze[17] that 2-nor PGE_1[18] and PGE_2 are markedly less potent than PGE_1 in inhibiting ADP stimulated platelet aggregation in rat platelet rich plasma (PRP) provided an early indication that prostaglandin activity is sensitive to the C_1-C_9 distance and to other minor carboxylic acid side-chain modifications. The methyl ester of 3-oxa PGE_1[19,20] (1), which was designed to block β-oxidation (one of the primary metabolic inactivating pathways of the natural prostaglandins), as well as other 3-[19,20], 4-[20], 5-[20], and 7-oxa[21-25] analogs have been reported to exhibit less activity than the natural congeners in a variety of *in vitro* and *in vivo* systems. Interestingly, 7-thia $PGF_{1\alpha}$ (2)[26], while an agonist in

some systems, inhibits placental C_{15}-prostaglandin dehydrogenase (PGDH). This finding, together with the reports that certain 7-oxa prostanoids[24] and 11-desoxy-8-carboethoxy PGE_1[27] analogs are competitive antagonists of the natural prostaglandins on isolated smooth muscle preparations, suggests that placement of a heteroatom/proton acceptor moiety in this area of the molecule transforms agonist activity into antagonist activity.



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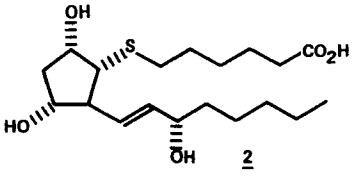
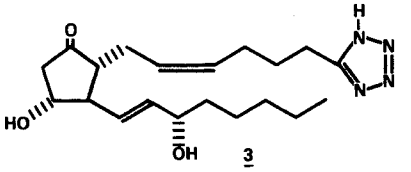


2

A series of 2-[28-30], 3-[29,30], 4-[29-31], and 5-[29,30,32] unsaturated prostaglandins has been found to exhibit somewhat reduced pharmacological activities. The activities displayed by a series of 2- and 3-substituted PGE_1 analogs[30] suggest that the smallest steric perturbation (e.g. 2-fluoro or 3-methyl) about the carboxylic acid moiety reduces activity while larger changes (e.g. 2-methoxy or 3,3-dimethyl) abolish activity. This finding has been extended to the 11-desoxy PGE_1 series[27] where, among the 2-, 3-, 4-, 5-, or 6-methyl analogs, the 5-methyl compound was found to be the most potent *in vitro* smooth muscle spasmogen.

The reduced activity displayed by 2-descarboxy-2-hydroxymethyl $PGF_{1\alpha}$[33] as well as 2-descarboxy-2-(tetrazol-5-yl) $PGF_{2\alpha}$ and $PGF_{1\alpha}$[34] suggests that prostaglandins of the F-series are sensitive to the nature of the C_1 moiety. In contrast, 2-descarboxy-2-(tetrazol-5-yl) PGE_1 and PGE_2 (3) possess activities[34] similar to the natural congeners. In light of the reduced activity displayed by the minor modifications mentioned above, the phenyl analog 4[35] provides an excellent example of the capriciousness of prostaglandin SAR. This compound possesses weak smooth muscle spasmogenic activity, poor (one-tenth PGE_1) reactivity on isolated C_{15}-PGDH but ten times the potency of PGE_1 in inhibiting collagen induced platelet aggregation in human platelet rich plasma.



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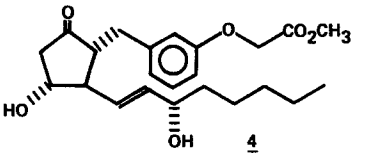
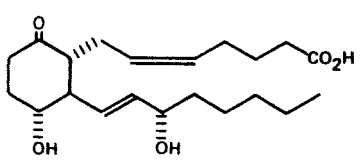


4

Cyclopentane Ring: As the activity of the natural prostaglandins differs widely with changes in number, type and stereochemistry of oxygenated substituents, it seems reasonable that cyclopentane ring analogs would exhibit different pharmacological profiles. The interesting concept of substituting a heteroatom for a ring carbon (and its pendant oxygen function) has been investigated with the synthesis of 8-aza[36,37], 9-oxa[38], 9-thia[39,40], 10-oxa[41], 11-oxa[42-44], 11-thia[45] and 9,11-dioxa[46] analogs. Unfortunately, except for extremely weak *in vitro* gerbil colon spasmogenic activity reported for the 11-oxa PGE_1, 11-thia PGE_1 and 9,11-dioxa PGE_1 little is known about these compounds.

Expansion of the cyclopentane ring into a suitably substituted cyclohexane ring[47,48] provided the PGE_2 analog 5 and the $PGF_{2\alpha}$ analog 6 which exhibit much less activity than the natural materials in several bioassays. Contraction of the cyclopentane ring afforded the novel cyclobutyl compound 7 which possesses[49] one-tenth the abortifacient activity in hamsters of $PGF_{2\alpha}$. Replacement of the cyclopentane ring with the

aromatic heterocyclic furan, oxazole and thiazole moieties[50,51] gave analogs such as 8 with weak rat uterus stimulant activity *in vitro* but which also inhibit swine lung C_{15}-PGDH.



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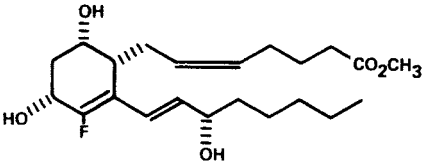

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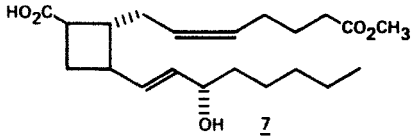

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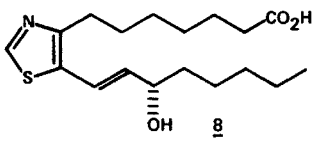
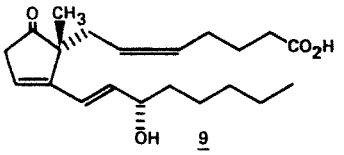


8

The chemical instability of PGEs[52] and PGCs[53] and the chemical as well as, in some species, enzymatic instability of PGAs[54] has prompted the design of more stable analogs. Among these analogs, 12-methyl PGA_2[55,56] possesses neither hypotensive (dog) nor gastric antisecretory activity in rats, 8-methyl PGC_2[57] (9) exhibits modest gastric antisecretory activity in rats, and 10,10-dimethyl PGE_1[58-61] displays negligible rat uterus stimulant activity *in vitro*. In contrast, 11-desoxy-11α-hydroxymethyl PGE_2[62-65] (10) exhibits rat uterus stimulant activity *in vitro* equivalent to $PGF_{2\alpha}$ with significantly reduced side-effects. These data suggest that increased steric bulk at carbons 8, 10 and 12 reduces activity of the natural congeners but that the C_{11}-C_{15} hydroxyl distance can be expanded to fit certain receptors with a concomitant increase in selectivity.



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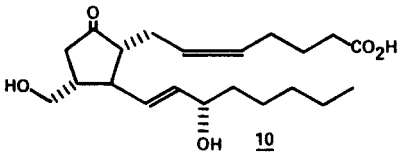

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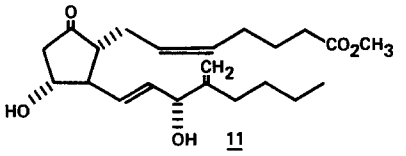
N-Amyl Carbinol Side-Chain: Initial reports of the activities displayed by the ω-alkyl prostaglandins[17,66,67] suggested that the n-amyl carbinol moiety could be successfully modified. Among the first and, to date, most successful analogs to be prepared are the 15- and 16-alkyl prostaglandins[19,68-72] which were rationally designed to be resistant to enzymatic C_{15}-dehydrogenation, the primary pathway of metabolic inactivation of the prostaglandins. Not only are these analogs resistant to C_{15}-oxidation as anticipated, but they also retain the smooth muscle spasmogenic activity of the natural prostaglandins[73]. Two members of this series, 15(S)-methyl PGE_2 and 16,16-dimethyl PGE_2, unlike PGE_2, possess potent oral gastric antisecretory activity in animals[74-76] and man[77-79] and many fold greater abortifacient activity in animals[80,81] and man[82]. However, side-effects (predominately gastrointestinal) observed during clinical evaluation of these compounds suggest that other activities may have been increased concomitantly with the increased uterine stimulant and gastric antisecretory activities. Recently, the 16-methylene analog 11 was reported[83] to exhibit a gastrointestinal profile in animals similar to 16,16-dimethyl PGE_2 yet to be somewhat better tolerated in

initial human studies. Confirmatory evidence regarding possible clinical advantages of this analog over the 15- and 16,16-methylated congeners has not yet appeared. Furthermore, the 16(R)-methyl analog **12** (ONO-464) has recently been claimed to possess potent oral hypotensive activity in dogs[84].



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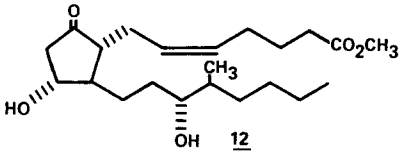
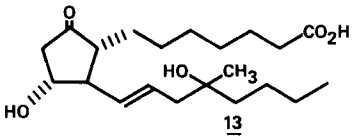


12

The finding that 13*α*,14*α*-methylene $PGF_{2\alpha}$[85-87] is not a substrate for C_{15}-PGDH whereas 13,14-didehydro $PGF_{2\alpha}$[88,89] inhibits C_{15}-PGDH represents another example of minor structural modifications resulting in a marked pharmacological change. In contrast, the 13*β*,14*β*-methylene $PGF_{2\alpha}$[85-87] and 14-chloro $PGF_{2\alpha}$[90,91] are substrates for the same enzyme.

As mentioned above, expansion of the normal 11*α*- to 15*α*-hydroxy distance provided the PGE_2 analog **10** possessing selective uterine stimulant activity *in vitro*. 15-Desoxy-16*ξ*-hydroxy PGE_1[92-95], which displays gastric antisecretory activity in the rat equivalent to PGE_1 and is a bronchodilator in the guinea pig, provides another example of this type of analog. Interestingly, the corresponding 17*ξ*-hydroxy and 15*ξ*-hydroxymethyl analogs are significantly less potent in both assays. Subsequent studies[96,97] found the 16-hydroxy-16-*methyl* analog **13** (SC-29,333) to possess 30-100 times greater gastric antisecretory activity than PGE_1 orally in dogs with only one-eighth the diarrheal effects in mice.



13

The observation that *ω*-bis-homo $PGF_{2\alpha}$[66,67] and the corresponding 17-oxa analog[98] were more active than $PGF_{2\alpha}$ as abortifacient agents in the hamster was followed by the preparation of a series of 16-phenoxy-*ω*-tetranorprostaglandins[99-101]. The profound abortifacient and uterine spasmogenic activity of these compounds was found to vary widely with both substitution of the phenoxy moiety and type of prostaglandin (Table I) in a manner which cannot be totally rationalized. Similar unexplained SAR differences, particularly in relation to prostaglandin type, exist with the closely related carbon isosteres, the 17-phenyl-*ω*-trisnorprostaglandins[102-104] (Table I). These closely related series do differ remarkably in one respect as the 16-phenoxy compounds are not substrates[105] for C_{15}-PGDH while the 17-phenyl congeners are substrates[106] for the same enzyme.

Additional studies with these analogs led to the findings that while PGE_2, 17-phenyl-*ω*-trisnor $PGF_{2\alpha}$, 17-phenyl-*ω*-trisnor PGE_2 and 16-(m-trifluoromethylphenoxy)-*ω*-tetranor $PGF_{2\alpha}$ differ widely in their abortifacient and *in vitro* uterine stimulant activity in rodents, all four compounds exhibit equivalent *in vivo* uterine stimulant activity in the rhesus monkey.[107] Furthermore, 16-(m-trifluormethylphenoxy)-*ω*-tetranor $PGF_{2\alpha}$ (fluprostenol)[15] and 16-(m-chlorophenoxy)-*ω*-tetranor $PGF_{2\alpha}$ (cloprostenol)[15] were found to be extremely potent agents for synchronizing estrus in horses[108] and cows[109], respectively, and are now

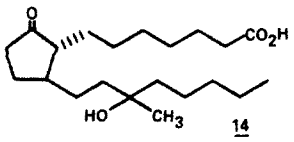
TABLE I

Compound[101,104]	Hamster Antifertility	*In Vitro* Uterine Contraction	Rhesus Monkey Uterus *In Vivo*
PGE_2	1	100	1
16-(p-chlorphenoxy)-ω-tetranor PGE_2	100	600*	-
17-phenyl-ω-trisnor PGE_2	4	112**	1.5
16-(p-fluorophenoxy)-ω-tetranor $PGF_{2\alpha}$	800	1000*	-
16-(m-trifluoromethylphenoxy)-ω-tetranor $PGF_{2\alpha}$	400	0.05*	1.2
17-phenyl-ω-trisnor $PGF_{2\alpha}$	360	300**	1

*Guinea Pig; **Rat

commercially available for this use. In contrast, initial human abortifacient studies[110] with fluprostenol suggest this compound to be only slightly more potent than PGE_2. The potency difference of these compounds among the various species underline the difficulty of selecting predictive models for various fertility control utilities. In addition to possible pharmacokinetic and metabolism arguments, species differences may, in part, be explained by the finding that prostaglandins exert their abortifacient effects in many non-primates by luteolysis[111]. Such a mechanism, however, has never been demonstrated in humans[112].

"Hybrid' Analogs: Chemists have begun to prepare prostaglandin analogs incorporating changes in several different parts of the molecule. An early example of this classical medicinal chemical SAR approach evolved from the observations that 11-desoxy-13,14-dihydro-15ξ-PGE_1 exhibited gastric antisecretory activity[113] and 11-desoxy PGE_1 displayed aerosol bronchodilator activity[114]. Subsequent modification of the n-amyl carbinol side chain[115-121] provided the methyl analog **14** which exhibits potent gastric antisecretory/antiulcer activity in rats but weak antifertility effects. Other investigators found the 16ξ-methyl analog[122] **15** to possess aerosol bronchodilator activity in guinea pigs ten times greater than PGE_2. The latter is particularly interesting in view of the lack of bronchodilator activity found in the closely related PGA_2. Another example of the "hybrid" approach is represented by the PGE_1 analog **16**[123] (TR-4197), which possesses gastric antisecretory activity five times that of PGE_1 in the rat but negligible smooth muscle, diarrheal or hypotensive activity.



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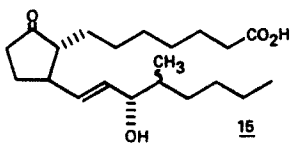

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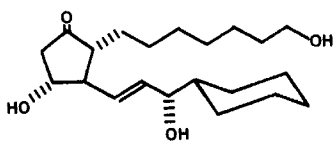


16

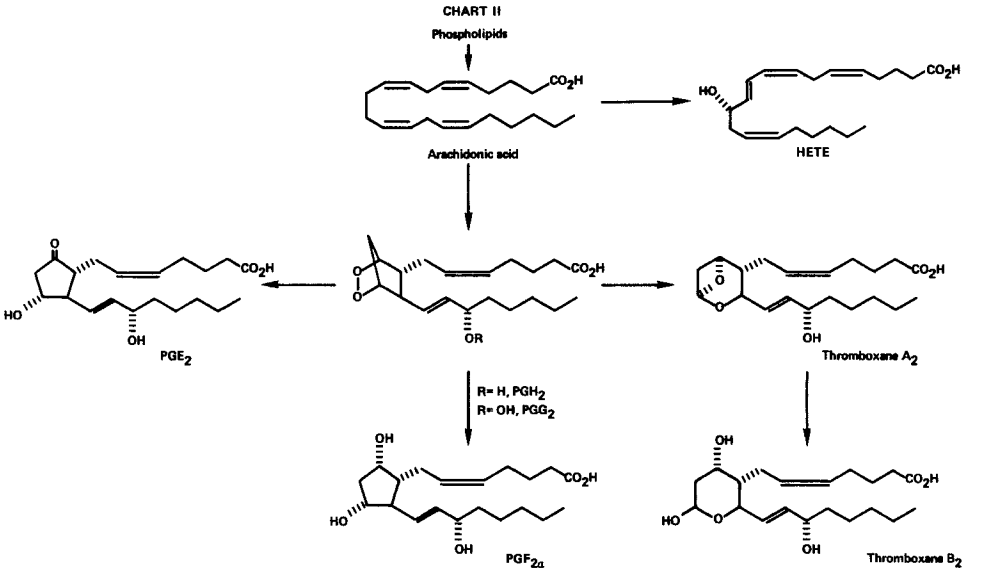
Considering the large number of modified prostaglandins being synthesized, the question arises whether, given the activities of two prostaglandins, the activity of the molecule combining both features can be predicted.

The data shown in Table II, which cannot be analyzed in any consistent manner, suggest that this will not be possible.

TABLE II

Compound[80,124]	*In Vitro* Contraction of Gerbil Colon	Rat Blood Pressure	Hamster Antifertility
PGE_1	1	1	1
4,5-cis PGE_1	0.1-0.3	0.1-0.3	0.05
15(S)-Me PGE_2	0.1	1	49
4,5-cis-15(S)-Me PGE_1	1-3.2	0.1-0.3	2

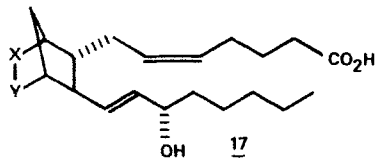
Biosynthesis and New Prostanoids: The endoperoxides PGG_2 and PGH_2 are a pivotal feature of the originally proposed[125-127] biosynthetic pathway of the natural prostaglandins (Chart II). These compounds were considered[128] to be transient intermediates converted by distinct enzymes under different stimuli and requiring different co-factors into either E- or F_α-prostaglandins. Isolation[129,130] of the endoperoxides, however, resulted in the finding that not only were they converted into the primary prostaglandins but possessed inherent pharmacological activities, including a possible key role[131-136] in platelet aggregation.



Continuing their pioneering work, Samuelsson and co-workers[137] have recently found that the versatile endoperoxides are converted by platelets into the labile ($T_{1/2}$ 30-40 sec) thromboxane A_2 which rearranges into thromboxane B_2 (Chart II). Initial reports indicate that thromboxane A_2 exhibits profound *in vitro* platelet aggregating activity[137] while thromboxane B_2 is correspondingly inactive. In addition, PGH_2 and thromboxane A_2 have been reported to be the constituents[138] of the previously isolated[139] rabbit aorta contracting substance (RCS). Other investigators[140] have reported that arachidonic acid is converted by platelets into HETE by a lipoxygenase independent of the endoperoxide biosynthetic pathway. HETE has been recently claimed[141] to be a potent neutrophil chemotaxic agent. These provocative findings suggest that the classical views of both prostaglandin biosynthesis and the physiological role of prostaglandins may have to

be revised. One may also speculate that other biological activities attributed to these new prostanoids will be forthcoming.

Based on these recent discoveries, endoperoxide (PGH_2) analogs have already been prepared as selective biosynthesis inhibitors (17a)[142], potent smooth muscle spasmogens (17b-e)[143-147] and stimulators of platelet aggregation (17b-e)[143-147]. Of particular note is the different activity profiles displayed by the double bond isomers 17a and 17e. These data suggest that endoperoxide analogs are subject to sensitive SAR changes similar to those found for prostaglandin analogs. As PGH_2 is relatively labile, these more stable analogs may also help to further define possible physiological roles for the endoperoxides.



17

a. X-Y; –CH=CH–, 5,6-dihydro
b. X-Y; –O–CH_2
c. X-Y; –CH_2–O–
d. X-Y; N=N
e. X-Y; –CH=CH–

Summary: Prompted by the potent pharmacological activities and acknowledged deficiencies of the natural prostaglandins, a wide variety of analogs have been prepared. As summarized above, animal studies with many of the modified prostaglandins suggest that some may be superior to the natural materials. However, with the exception of the 15- and 16-methylated prostaglandins little has been reported concerning the therapeutic effects of prostaglandin analogs in man. Extrapolating from the past, one may anticipate both an accelerated preparation and clinical evaluation of analogs. From these studies should emerge modified prostaglandins that will find their way into the therapeutic armamentarium to be used against many disease states.

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Section III - Chemotherapeutic Agents

Editor: George B. Whitfield, The Upjohn Company, Kalamazoo, Michigan

Chapter 10. Antibiotics

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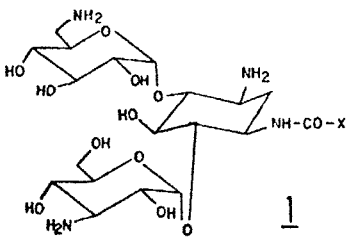
General. Abstracts from symposia were published on recent advances in β-lactam antibiotics[1]; antibiotic sugars[2]; a series of 17 antimicrobial symposia, 34 antimicrobial sessions and a symposium on sisomicin[3]; laboratory evaluation of aminoglycoside antibiotics, new semi-synthetic penicillins and cephalosporins[4]. Proceedings of conferences on cephalexin and cephaloridine[5], gentamicin[6], and amoxicillin[7] and a symposium on antibiotic research appeared[8]. Abstracts were published on a conference on biosynthesis, chemistry, new antimicrobials, clinical and pharmacological effects, mechanisms of action, pharmacokinetics, and comparison of the cephalosporins[9]. Reviews appeared on new antibiotics[10], chemical and biological studies on 16-membered macrolide antibiotics[11], therapy of anaerobic infections[12], and antibiotic resistance plasmids of S. aureus and their clinical importance[13]. The effects of modifications on the properties of aminoglycoside antibiotics containing 2-deoxy-streptamine[14] and recent developments and classification of antibiotics according to chemical structure were reviewed[15]. Books were published on resistance to aminoglycoside antibiotics[16], infection with anaerobes[17], drug resistance[18], antimicrobial agents in medicine[19], and a review of microbiology in 1975[20]. Two new journals appeared in 1975; European Journal of Applied Microbiology[21], and the Journal of Antimicrobial Chemotherapy[22].

Aminoglycosides. Sisomicin was studied against 565 gram-negative and gram-positive clinical isolates[23]. Except for Serratia marcescens, over 90% of isolates of the gram-negative bacilli were inhibited by 1.56 mcg/ml or less. In another study,[24] sisomicin was found to be very effective in vitro compared with gentamicin and tobramycin. It was more active on a weight basis than gentamicin against Pseudomonas and Klebsiella species and indole-positive Proteus[25]. Gentamicin was more active than sisomicin against E. coli, Serratia species, Enterobacter, and P. mirabilis. Both antibiotics were active against methicillin-resistant strains of S. aureus. In a comparative study of the pharmacokinetics of sisomicin, gentamicin and tobramycin[26], sisomicin had the highest serum concentrations.

Gentamicin was the most efficacious antibiotic against P. aeruginosa or S. aureus among 47 patients with a diagnosis of mucoviscidosis[27]. In an in vitro study of amikacin, gentamicin and aminodeoxybutirosin against Proteus and Pseudomonas[28], no significant differences were found as related to their blood levels. Amikacin was the most active against S. marcescens. The effects of gentamicin on albumin binding of bilirubin transport were studied[29] and no significant interactions between the substances were observed. Gentamicin complex and C_1 were equally effective in complicated urinary tract infections[30] and pharmacokinetics were similar. Tobramycin appeared to be as efficacious as gentamicin in the treatment of serious P. aeruginosa infections in 25 hospitalized patients[31]. Tobramycin may have a similar type of activity to that of gentamicin and is believed to cause mis-reading in protein synthesis[32]. Gentamicin and tobramycin were active against Mycoplasma hominis[33].

Amikacin was effective[34] but high frequency hearing loss[35] may limit its use. Use in neonates was contraindicated[36]. It was weaker than gentamicin, dibekacin or tobramycin in in vitro studies[37], although against several strains of P. morganii and P. aeruginosa, amikacin had lower MIC's. Twenty-three amikacin analogues of kanamycin A with various acyl residues were prepared (1a-f) and their activities compared[38]. The presence of a terminal basic amino group in the side chain is most important for antibacterial activity. Removal of the terminal basic function lowered the activity and removal or modification of the α-hydroxy group resulted in a considerable decrease of activity. Lack of both the basic amino and α-hydroxy groups made the analogues inactive. A new semi-synthetic analogue of sisomicin, Sch 20569, has been prepared by a novel process and found to possess improved activity against resistant bacteria[39]. Studies were carried out in comparison with gentamicin, tobramycin and amikacin against a variety of aminoglycoside-sensitive gram-negative and gram-positive bacteria[40]; protection tests using experimental infections in mice and ataxia studies in cats relative to other aminoglycosides were compared[41]. Sch 20569 and amikacin showed markedly greater activity than gentamicin against E. coli, Klebsiella, Enterobacter, Citrobacter, and indole-positive Proteus[41]. Of 93 gentamicin-resistant strains tested, 57 were sensitive to Sch 20569 and 58 to amikacin.



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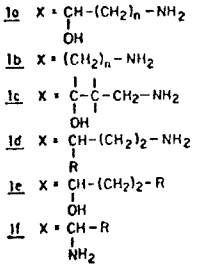
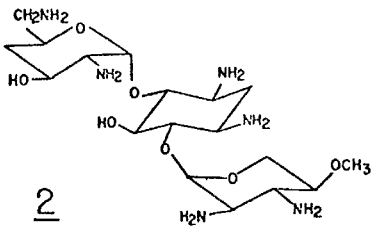


1a X = CH-(CH2)n-NH2 (OH)
1b X = (CH2)n-NH2
1c X = C-C-CH2-NH2 (OH)
1d X = CH-(CH2)2-NH2 (R)
1e X = CH-(CH2)2-R (OH)
1f X = CH-R (NH2)



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Verdamicin[42], has an in vitro and in vivo spectrum similar to gentamicin and sisomicin. New aminoglycoside antibiotics designated seldomycin factors 1, 2, 3 and 5 are produced by Streptomyces hofuensis[43]; factor 5 is the most active. The structure of factors 1, 2 and 5 were reported[44,45]. The latter (2) contains an unusual modified pentopyranose. Two new aminoglycosides, JI-20A and JI-20B, produced by Micromonospora, were reported[46]. The structures of the gentamicin antibiotics A_1, A_3 and A_4 were elucidated[47], as was the structure of gentamicin A_2[48]. Gentamicin oxazolidine derivatives formed by the condensation of aldehydes with gentamicin were reported[49]. 4'-Deoxykanamycin A was prepared[50] which has improved anti-Pseudomonas activity compared to kanamycin A. The total synthesis of dihydrostreptomycin[51] and 6'-N-alkyl derivatives of 1N-[(S)-4-amino-2-hydroxybutyryl]-kanamycin[52] were reported. The latter is only slightly affected by 6'-N-acetyltransferase. 3'-Deoxybutirosin B was synthesized[53] and is more active than butirosin B or 3',4'-dideoxybutirosin B. 5"-Amino-3',4',5"-trideoxybutirosin A[54,55] showed enhanced antibacterial activity against those P. aeruginosa and E. coli which are resistant to butirosin or gentamicin. The synthesis of both α- and β-anomers of methyl-gentosaminide, a component of gentamicin A and 66-40B, was described[56]. The absolute structural configurations of destomycin A, B[57], and C[58] were elucidated. Paromomycin derivatives prepared by protecting the 6"-amino group were reported[59]. The 1-N-acyl derivatives

of 3',4'-dideoxy-6'-N-methyl-kanamycin B, which inhibit resistant strains producing 6'-N-acetyltransferase were described[60]. 1-N-(α-substituted ω-aminoacyl) derivatives of lividomycin A were prepared[61] which are effective against lividomycin A-resistant strains of E. coli. The probable structure of fortimycin A, produced by Micromonospora olivoasterospora[62] was described. The total synthetic approach to antibiotic monosaccharides was discussed[63]. The preparation of stable gentamicin adenylyl transferase[64] and gentamicin acetyl transferase I[65] were reported. A new aminoglycoside 3'-phosphotransferase III, was reported[66]. The structural requirements of the substrate for the action of the aminoglycoside 6'-N-acetyltransferases were described[67].

β-Lactams, Cephalosporins and Penicillins. A new orally-active cephalosporin derivative named cefetrizine (SKF 60771; BL-S640) was reported[68-70] which appeared comparable in vitro to cephalexin but was poorly bactericidal using high inocula[71]. Its spectrum was similar to the other cephalosporin analogues and cross-resistance was observed with cephalexin, cephalothin and cefazolin[72]. It demonstrated the best activity when compared to cephalothin and cephalexin against 338 clinical isolates of nearly all microbial species studied except for Haemophilus influenzae and Diplococcus pneumoniae, against which cephalothin was slightly more active[73,74]. In mouse infections, cefatrizine was highly effective against a variety of pathogenic bacteria and, in comparison with other cephalosporins, the in vivo activity was greater than would have been predicted by in vitro activities[75]. Clinically, the majority of 32 patients with urinary tract infections responded well[76]. In normal volunteers, it compared unfavorably with the other cephalosporins by oral and intramuscular administration[77]. At attainable serum levels of 10 μg/ml, both cefoxitin and cefamandole were bactericidal for nearly all strains of E. coli, Klebsiella, P. mirabilis and S. aureus but were inactive against P. aeruginosa and enterococcus[78]. Cefotoxin was highly active against B. fragilis and most other anaerobes at ≤32 μg/ml[79]. Compared with other β-lactam antibiotics and amikacin, cefoxitin was found to have less activity against Serratia marcescens than carbenicillin but greater activity than ampicillin[80]. Overall response of 18 patients treated with 3-6 grams daily iv of cefoxitin was very good and no side effects were seen[81]. Cefamandole was active against high and low inocula of penicillin-sensitive and β-lactamase-producing strains of S. aureus and various strains of Proteus sp.[82]. Cefamandole nafate, the formyl ester of cefamandole, is microbiologically equivalent to and readily converted into cefamandole in vitro[83]. The pharmacology of cefamandole and cephalothin is similar but iv blood levels of cefamandole are higher[84]. Protein binding is similar to cephalothin and there is no evidence of metabolic degradation in vitro[85]. Cefamandole was effective in the treatment of gram-negative pneumonia[86], in soft tissue, respiratory and urinary tract infections[87,88]. It was active in vitro against most clinical isolates and human pharmacology in 10 patients was reported[89]. Cefazolin was studied in vitro against dense populations of enterobacteria[90] and compared with cephalothin in patients for degree of phlebitis production[91].

A new cephalosporin, cefuroxime, has increased stability to β-lactamases[92]. Injections by iv and im routes were well-tolerated and there was no absorption after oral administration[93]. Ceftezol (CG-B3Q), related to cefazolin, gave a clinically satisfactory response in 85% of gram-positive infections and 65% against gram-negative infections[94]. A parenteral cephalosporin, SKF 59962, was found equal to or superior in vitro and in vivo to cefazolin and cephalothin[95,96].

New semi-synthetic penicillins were evident. The chemistry, structure activity relationship, antimicrobial activity and biological properties of PC-455, an N-acyl derivative of amoxycillin, was reported[97,98]. Protein binding of 70% was noted and its activity is 5-10 times that of carbenicillin against Pseudomonas and Klebsiella. Improved activity equal to 4-fold that of carbenicillin against Pseudomonas was reported for CP-33,994; however it was inactive against ampicillin-resistant strains of E. coli[99-101]. A new semi-synthetic penicillin derived from aminobenzylpenicillin, PC-904, was also reported to be more active than carbenicillin against Pseudomonas but was inactive against β-lactamase-producing Staphylococci[102]. Absorption, tissue distribution and excretion were studied[103]. Bacampicillin, an orally well-absorbed derivative of ampicillin was found to be better absorbed than ampicillin in animal studies and more effective in experimental infections[104,105]. Studies with bacampicillin in humans produced serum levels 3 times as high as ampicillin itself, since it quickly liberates ampicillin as the active metabolite after oral administration[106]. A new ureido-penicillin (Bay e 6905) was studied against Pseudomonas in vitro[107] and in the clinic[108]. Carfecillin, a new orally-administered ester of carbenicillin was sucessful in eradicating the pathogen in 21 of 35 cases of urinary infections (60%) and side effects were virtually absent[109]. A new single β-lactam antibiotic, FR-1923, produced by a new Nocardia species, is active against gram-negative bacteria, including Pseudomonas, in vitro, but is relatively inactive against gram-positive bacteria[110,111].

In clinical trials, oral cyclacillin was found to give 4-5 times peak ampicillin levels[112]. Ampicillin was significantly less active than amoxycillin in mouse infections when administered by oral and parenteral routes[113]. Ampicillin gave lower serum levels after oral administration to fasting men and women than did amoxycillin although levels were higher than epicillin[114]. Ampicillin was compared to cephalexin for shigellosis in infants and children[115]. Ticarcillin was studied alone and compared with carbenicillin and ampicillin in a series of in vitro and clinical studies, particularly with Pseudomonas organisms[116-120]. The in vitro activity of the semi-synthetic penicillin BL-P1654 was reported[121,122] although it has been withdrawn from clinical trials because of nephrotoxicity in animals[123]. Mecillinam, formerly called FL1060, a 6β-amidinopenicillanic acid derivative, showed higher activity than ampicillin against clinical isolates of Enterobacteriaceae[124] and activity against certain Klebsiella and Proteus strains resistant to ampicillin[125]. Pivmecillinam (FL1039) was shown to be a safe and potent antibiotic in the treatment of urinary tract infections[126]. Penicillin V was effective in 30 out of 32 women with uncomplicated urinary tract infections[127]. Six penicillins and seven cephalosporins were compared against 118 clinical isolates of S. aureus and methicillin and nafcillin were found to be most stable to staphylococcal β-lactamase; benzylpenicillin and cephaloridine were most susceptible[128]. Neither cephaloridine, cephalothin or cefazolin in combination with gentamicin produced any greater nephrotoxicity in rats than gentamicin alone[129].

Macrolides. Higher serum concentrations of erythromycin were produced by erythromycin estolate then erythromycin stearate or base[130] in one study and in another[131], erythromycin estolate was no more effective than the base despite higher total serum concentrations. 9-(S)-Dihydroerythromycin A, an acid-stable erythromycin with good antibacterial activity was prepared[132]. A new compound, the L-aspartic acid salt of erythromycin A cyclic carbonate has low toxicity and twice the activity of erythromycin[133]. A series of 4"-O-sulfonyl derivatives of erythromycins A, B and C[134], 4"-deoxy-4"-oxoerythromycin B derivatives[135], and

11-substituted erythromycin B derivatives[136] were described. Novel megalomicin A ester derivatives have been prepared[137]. Absorption, distribution and excretion of ^{14}C-josamycin base and propionate were studied in rats[138]. Oral administration of ^{14}C-josamycin propionate rapidly produced very high plasma and tissue concentrations in lung, liver, kidney and spleen. In vitro and in vivo studies of josamycin[139] indicated that the antibiotic was well tolerated. The in vitro effectiveness of josamycin compared to several other antibiotics indicated that 6 of 7 methicillin-resistant S. aureus coagulase (-) endocarditis isolates were sensitive to josamycin and all were resistant to erythromycin[140]. Josamycin was as effective as clindamycin against anaerobes but less effective than ampicillin and erythromycin against enterococci[141].

Rifamycins. Halomicin A, B and C can be converted to rifamycin S, which may then be converted to rifampicin[142]. Halomicin C can be converted to an analogue of rifamycin S, which by analogy, may be converted to a rifampin analogue. Rifamycin S can be produced by hydrolyzing rifamycin O in a heterogeneous system with a minimum amount of acid[143]. New processes for production of rifamycins by Micromonospora have been described; the production of rifamycin SV by M. ellipsospora[144], the preparation of rifamycins S and SV by M. chalcea[145] and, utilizing two new species of Micromonospora, an antibiotic mixture consisting of 3-methylthiorifamycin SV and rifamycin SV were produced[146]. Utilization of rifampicin in the treatment of leprosy in patients whose disease was getting worse was reported[147] and the successful treatment of diphtheria with rifampin was described[148]. Four strains of S. aureus isolated from tuberculosis patients on rifampin therapy were rifampin-resistant but of 32 strains isolated from patients not on rifampin, 31 were susceptible to the antibiotic[149].

Tetracyclines. Doxycycline was effective in typical ear, nose and throat infections[150]. Minocycline was highly active against Herellea vaginicola[151] and clinical isolates of Acinetobacter[152]. In clinical prophylactic treatment with minocycline, moderate to severe vestibular reactions were common. A new process was described[153] to obtain 6-methylenetetracyclines and the 6-deoxytetracyclines. The total synthesis of tetracycline antibiotics beginning with 3,4,10-trioxo-1,2,3,4,4a,9,9a,10-octahydroanthracenes was described[154]. Compounds of 6-deoxytetracycline and methionine having high aqueous solubility, improved stability, and high blood levels were described[155]. 6-α-Deoxytetracyclines were produced by single stage dehalogenation and hydrogenation of the corresponding 6-methylene-11-α-halo-12-oxo compounds[156], as were 6-methylene tetracyclines produced by reductive dehalogenation of 11-α-halo-12-oxo compounds[157].

Clindamycin. In aerobic and/or anaerobic infections treated with parenteral clindamycin, 121 of 122 patients responded to a single course of therapy[158]; severe diarrhea was found in only 2 patients. Of 41 patients with a variety of infections, no significant side effects were observed with parenteral clindamycin[159]. At another center, however, contradictory results were obtained[160]. Of 1000 consecutive patients who received clindamycin, 66 developed diarrhea which was unrelated to route of administration or duration of therapy. Gastrointestinal effects were experienced by 582 patients. Although the incidence of GI side effects were high, the morbidity from them was minimal. Ten cases of protracted diarrheal illness after the oral administration of lincomycin or clindamycin in standard dosages were observed in previously healthy subjects[161]. Thirty patients undergoing total hip replacement received clindamycin

beginning the day before surgery and showed mean serum levels of 7.33 µg/ml (2.63 µg/gm) in bone[162].

The MIC of lincomycin and clindamycin for strains from multiple serogroups of streptococci was determined. From groups A, B, C, F, G, H, L, and M, the MIC ranges from 0.02 to 0.39 µg/ml for lincomycin and ≤0.01 to 0.9 µg/ml for clindamycin. Streptococcus faecalis was resistant to both antibiotics and occasional strains of non-group D were highly resistant to both antibiotics[163]. Whereas all pretreatment isolates of S. epidermidis from the anterior mares were susceptible to clindamycin, 6 of 9 post-treatment isolates were resistant[159]. Clindamycin acted synergistically with gentamicin against 3 of 6 strains of Streptococcus viridans[164] and the combination was found effective in the treatment of anaerobic infections[165].

Resistance. Methicillin-resistant S. aureus strains were isolated from 23 patients during one 12 month period[166]. In another hospital study, oxacillin-resistant S. aureus was isolated from six patients during one 5 month period[167]. Cefazolin, cephaloridine and cephalothin were inactivated by methicillin-sensitive and methicillin-resistant strains of S. aureus although the rates were quite different[168,169]. Exposure of Klebsiella sp. and S. aureus to either cephalothin or cefoxitin resulted in simultaneous increase in intrinsic and β-lactamase-mediated resistance[170]. Pseudomonas aeruginosa KM 338 was found to exhibit intrinsic resistance to penicillin due to the development of a permeability barrier[171]. Serratia marcescens isolates were tested for susceptibility to cephalothin, carbenicillin, ticarcillin, ampicillin and cefoxitin and it was determined that β-lactamase production was not the sole determinant in resistance[172]. The biochemical properties of a penicillin β-lactamase mediated by an R-factor from Bordetella bronchiseptica was described[173]. Carbenicillin resistance and pyocinogenic factors were shown to be transferred by conjugation in P. aeruginosa[174]. Four sensitive Haemophilus influenzae type B strains were transformed to ampicillin resistance with isolated plasmid DNA preparations[175]. Other investigators found that transfer of ampicillin resistance between strains of H. influenzae is probably mediated through conjugation[176].

Evidence for plasmid-mediated tetracycline resistance was found in 92 (84%) of 110 hospital strains of tetracycline-resistant E. coli[177]. Twenty seven (13.5%) of 200 penicillin-resistant staphylococcal isolates and 14.5% of 511 enterobacterial isolates were tetracycline-resistant but minocycline-sensitive[178]. Changes in resistant flora were studied and, with tetracycline, there was a mean increase of 10^4 resistant strains per gram compared to 10^1 per gram with doxycycline[179]. Tetracycline was shown to be an active inducer of resistance in P. aeruginosa involving an enzyme responsible for the active transport of tetracycline into the cell[180]. Strains isolated from hospital patients showed 60% of 102 Shigella sonnei resistant to both ampicillin and tetracycline while 14 S. flexneri strains showed no ampicillin resistance but 50% resistance to tetracycline[181]. Streptococcus faecalis strain DS-5 and S. pyogenes strain AC-1 both have a 17 million dalton plasmid that determines resistance to erythromycin, lincomycin and vernamycin B; and erythromycin-resistance is inducible in AC-1 and constitutive in DS-5[182]. Six of 10 strains of β-hemolytic streptococci lost their resistance after cultivation at 41°C or addition of acriflavine (0.2 µg/ml) indicating plasmid control[183].

Resistance to gentamicin among hospital isolates of P. aeruginosa increased from 13.9% in 1969 to 38.9% in 1972 in one hospital study[184]; however of 102 clinical isolates of Serratia marcescens, most fell

within therapeutic range MIC's for gentamicin[185]. A study of S. aureus strains showed a greater increase in MIC values for kanamycin and amikacin than for gentamicin, sisomicin or tobramycin[186]. Aminoglycoside 6'-N-acetyl transferase[187,188] and 3'-phosphotransferase I and II in P. aeruginosa[189] were characterized. The genetic properties of an R-factor carrying resistance to aminoglycoside antibiotics was described[190]. A third type of P. aeruginosa plasmid confers resistance to gentamicin by gentamicin acetyl transferase I and to kanamycin and neomycin by neomycin phosphotransferase I[191]. An R-factor-mediated acetylating enzyme was characterized from a clinical isolate of P. aeruginosa which inactivates amikacin[192]. R-factors determining multiple resistance including both gentamicin and carbenicillin have been identified in high incidence among hospital isolates of P. aeruginosa[193,194]. Shigella dysenteriae strains isolated during an epidemic were found to contain two different R-factors, one causing resistance to chloramphenicol, tetracycline, streptomycin and sulfonamides and one causing ampicillin resistance[195]. The control of transfer of resistance plasmids in E. coli K-12 and clinical isolates of E. coli was studied[196] and the genetic basis of multiple drug resistance of Neisseria gonorrhoeae was investigated by the technique of transformation and six different loci were characterized[197].

Aminoglycoside-β-lactam synergy. Of 264 recent clinical isolates of P. aeruginosa tested in vitro using tobramycin and gentamicin alone and in combination with carbenicillin, gentamicin-carbenicillin was synergistic against 57% of the strains and tobramycin-carbenicillin against 46%[198]. With gentamicin added to therapeutically achievable concentrations of carbenicillin, ticarcillin or BL-P1654, the inhibitory and bactericidal activity of all three penicillins was enhanced for the majority of isolates. Inhibition of isolates highly resistant to gentamicin was not improved by combining the penicillins with gentamicin[199]. The in vitro inactivation of gentamicin by carbenicillin and ticarcillin has been shown again and a 25% to 74% reduction in half-life has resulted from the concomitant administration of carbenicillin and ticarcillin[200]. Gentamicin, tobramycin and amikacin were studied alone and in combination with cephalothin against Klebsiella[201]. Sensitive organisms showed increased inhibition in combination with cephalothin; isolates highly resistant to the aminoglycoside seldom demonstrated increased inhibition by the combination. In 186 cancer patients treated with combinations of ticarcillin-tobramycin, ticarcillin-cephalothin, and tobramycin-cephalothin, the three antimicrobial regimens were similarly effective and resulted in a favorable response in approximately 55% of the patients[202]. In potentially leukopenic patients with neoplastic disease, the combination of amikacin-carbenicillin or gentamicin-carbenicillin was equally efficacious[203]. Amikacin alone might not be the optimal therapy for gram-negative bacteremia in non-granulocytogenic patients and the use of potentially synergistic combinations should be considered[204]. Clinical strains of enterococci were studied for susceptibility to oxacillin and gentamicin alone and in combination. At attainable blood levels, the combination was synergistically bactericidal against 27 (80%) strains[205]. Nafcillin plus gentamicin was synergistic against 13 of 14 clinical isolates of enterococci, oxacillin plus gentamicin against 3 of 14 strains and methicillin plus gentamicin against only 1 of 14 strains[206]. In another study[207], 57 clinical isolates of enterococci were tested for sensitivity to 10 single antibiotics and the combinations of ampicillin-amikacin and ampicillin-rifampicin. Gentamicin was the most active followed by tobramycin, amikacin, kanamycin and streptomycin. Amikacin and ampicillin were synergistic; ampicillin and rifampicin were antagonistic. In vivo studies[208], showed no synergy between parenteral amidinopenicillin and erythromycin, oxytetracycline or carbenicillin. With

Streptococcus viridans strains, synergy was demonstrated with penicillin and streptomycin or gentamicin with 4 of 6 strains. Clindamycin with streptomycin or gentamicin acted synergistically with 3 of 6 strains[209]. Synergy with the penicillin-gentamicin combination was found in 2 of 9 strains of Streptococcus mutans; addition effects occurred in 7 of 9 instances and antagonism occurred in 8 instances with clindamycin-gentamicin combinations[210]. Nafcillin was evaluated in combination with gentamicin, vancomycin, oxacillin, chloramphenicol and clindamycin in vivo against S. aureus infections[211]. The most protective antibiotics singly administered were gentamicin or clindamycin followed in decreasing order by nafcillin, vancomycin, chloramphenicol and oxacillin. Most of the combinations were beneficial and were additive, the best combination being gentamicin-nafcillin. Sisomicin was equally as effective as gentamicin.

Miscellaneous. Novel antibiotics in the everninomicin family, everninomicin I and hydroxylaminoeverninomicin D were described[212]. A uniquely synergistic broad spectrum bactericidal antimicrobial combination MK641/MK642, designed for oral or parenteral use at fixed ratios, has been reported[213-216]. MK641 is 3-fluoro-D-alanine; MK642 is sodium D-4 (2-oxo-3-penten-4-yl)-amino -3-isoxazolidinone hemihydrate, a cycloserine analog. The combination acts by sequential blockade of cell-wall biosynthesis.

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Chapter 11. Antifungal Agents.

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Reviews - A comprehensive review of the antifungal drugs presently in clinical use summarised their developmental histories, modes of action and clinical applications. [1] The proper evaluation of new antifungal drugs was considered to be of utmost importance. The difficulties in evaluating the place of flucytosine (5-FC) from reading published data was quoted as an example.[2] The present status of the polyene antibiotics, their modes of action and toxicity were reviewed.[3] Amphotericin B was considered in greater detail, especially the importance of combined therapy with other drugs and the potential immunological role resulting from its adjuvant properties. The outcome of treatment with amphotericin B was not solely dependent on its antifungal properties.[4] In a review on oculomycosis, 61 ocular fungal pathogens were considered. [5] The results of antifungal susceptibility were recorded and their application to 25 cases discussed. Although pimaricin was the first line therapy, the use of amphotericin B, 5-FC, clotrimazole, miconazole, econazole (R14827) and thiabendazole was considered.

The mode of action of polyene antibiotics and the role of fungal sterols were reviewed and the importance of their study regarded, not just with respect to antifungal activity but also in the understanding of membrane function.[6] Polyene resistant mutants as a potential source for new sterols is considered in the same review.

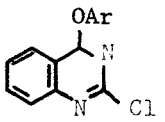
Laboratory Methods - Methods for susceptibility testing of yeasts and fungi in diagnostic laboratories were clearly described.[7] The same article described the assay of antifungal agents in biological fluids using a seeded agar plate diffusion technique. 5-FC was assayed in the presence of amphotericin B using yeast nitrogen broth with 1% agar added - a medium through which amphotericin B does not diffuse.[8] The rapid assay of 5-FC in 15 minutes was achieved using a high pressure liquid chromatography column.[9]

A surface inoculum on 0.45μ membranes placed on drug-containing solid media was used in determining susceptibility to antifungal drugs. Membranes on which growth was inhibited were washed and placed on drug-free medium to establish fungicidal levels.[10] The difficulties in comparing published studies of antifungal activity were discussed and data presented on the susceptibility of *Coccidioides immitis*, *Candida albicans* and *Cryptococcus neoformans* to amphotericin B, 5-FC and clotrimazole determined under the same conditions.[11] The *in vitro* susceptibilities of

strains isolated from eight patients with chromomycosis were measured. In two cases resistance was demonstrated to 60 and 100 μg/ml.[12]

In Thayer-Martin medium, amphotericin B was more effective than nystatin in suppressing C. albicans and remained stable for up to 14 days at +4°C. [13] Activity of amphotericin B was rapidly lost in GYE and Salvin liquid media. [14]

New Antifungal Agents - A new species of Actinoplanes (NRRL.5325)produced a polyene antifungal antibiotic, Sch. 16656, having one major and three minor components. [15] The major component, a heptaene, was active in vitro and in vivo against C. albicans and Torulopsis glabrata. Econazole (R14827), an imidazole derivative, was active against dermatophytes, yeasts, dimorphic fungi, aspergilli, mycetoma-causing agents and many gram-positive bacteria. Its toxicity was low and was effective both orally and topically against dermatophytic and candida skin infections of guinea pigs.[16] 2-Imidazolyl-1-indanol and 2-imidazolyl-1-tetralol and derivatives halogenated at the benzene nucleus had antifungal activity.[17] Thirty one new esters of 7-nitro 8 hydroxyquinolone were prepared. Of these, 1 benzoic, 1 benzoic isoester, 1 cinnamic, 2 naphthoic and 2 furan esters showed significant activity against Trichophyton mentagrophytes and C.albicans together with a low acute toxicity when given intraperitoneally to mice.[18] Similar activity was shown in the 1-4-naphtho-quinones. [19] A series of new aryloxyquinazolines were prepared from 2, 4-dichloroquinazoline and the appropriate mono to penta substituted phenols.



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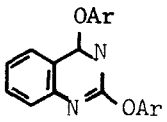
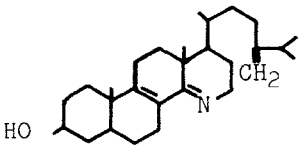


II

Forty one were more active than phenol against C. albicans, C.tropicalis, C.neoformans, Rh.mucilaginosa and G.candidum. Compounds T1456 (1, AR=pNO_2- C_6H_4) and T1514 (11, Ar = C_6 Cl_5) were most effective.[20]

A new antimicrobial agent, triethyl-n-hexylammonium triethyl-n-hexylbromide was active against C. albicans and gram-positive bacteria.[21] It had a disruptive action on cell membrane and was considered as a possible disinfectant. One major and six minor structurally-related components comprise new azasteroidal antifungal antibiotics, A25822 A-F obtained from Geotrichum flavo-brunneum. [22] The major factor was A25822B.[23]



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It was active against fungi and yeasts including C. albicans, T.mentagrophytes, C. neoformans and Histoplasma capsulatum, but had limited activity against bacteria. Absorption occurred from the gut giving serum levels within the therapeutic range. Antagonism was demonstrated with the polyene antibiotics. [24] A product of Sorangium, myxin, had a wide antifungal spectrum but excluding the dermatophytes and aspergilli. [25] A9145, an adenine-containing antibiotic was highly effective against C. albicans in vitro and in experimental infections. [26] A thiocarbamate derivative KC9147 in a 1% solution compared favourably with tolnaftate in experimental infections of T. asteroides and M. canis in guinea pigs.[27] Evidence of bacterial inhibition of C. albicans by Escherichia coli [28] and Bacillus subtilis [29] was demonstrated. The inhibition was also found with dialysates of bacterial cultures.

Biological and Chemical Studies - The production of candicidin from Streptomyces griseus was studied and the controlling role of inorganic phosphate, which increased mycelial growth while decreasing candicidin production, shown. [30] The sequential formation of the antibiotic complex was elucidated and the glycosylation of the macrolide ring shown to occur during the secretion process.[31] The primary effect of candicidin in yeast cells was on components related to ion transport across the cell membrane.[32] This effect could be neutralised by pre-incubation with a solution containing KCl and MgCl. A similar effect was observed with amphotericin B, the methyl ester and N-succinyl fungimycin but not with filipin.[33] Potassium leakage from C. albicans induced by polyene antibiotics was greater in the logarithmic as opposed to the stationary growth phase. [34] Amphotericin B inhibited photosynthetic-electron transfer in chloroplast membranes from maize, peas and Euglena gracilis. [35] The relationship of membrane sterols was studied; amphotericin B methyl ester was more active against organisms whose main membrane sterol was ergosterol, whereas cholesterol was more important for filipin action. [36] The lower susceptibility of Saccharomyces cerevisiae to nystatin at higher temperatures was related to a lower ergosterol content at these temperatures.[37] Mutants of C. neoformans obtained by exposure to ultra violet rays were obtained, resistance to amphotericin B, nystatin and pimaricin was related to changes in the sterols - Δ^{7}-22-ergostadien - 3β-ol and ergosterol. [38]

The changing sensitivities of C. albicans, Aspergillus fumigatus and H. capsulatum during various phases of growth were studied. C. albicans was more susceptible to amphotericin B methyl ester (AME)[39] miconazole, and clotrimazole [40] during the logarithmic growth phase. Germinating conidia of A. fumigatus were sensitive to amphotericin B methyl ester; ungerminated conidia were resistant. [41] The mycelial phase of H. capsulatum was more susceptible than the yeast phase to amphotericin B but the reverse applied with actinomycin D. [42]

The effect of miconazole on C. albicans as seen by electron microscopical examination of thin sections was a progressive degradation of the plasmalemna. Increasing concentrations resulted in dissolution of the

plasmalemna, alterations of all cytoplasmic organelles and loss of cell shape.[43] Scanning electron microscopy studies of C. albicans showed that fungistatic levels of miconazole produced rough surfaces with multiple disorientated buds and bud scars.[44]

The susceptibility of 390 strains of C. albicans to the 5 fluoropyrimidines - flucytosine (5-FC), 5-fluorouracil and 5-fluorouridine were tested and 6 different phenotypes recognised.[45] Resistance to 5-FC was due to a deficiency of uridine monophosphate phosphorylase or cytosine deaminase.[46] Examination of a further 29 strains resistant to 5-FC showed that the de novo synthesis of surplus pyrimidines may occur.[47]

Amphotericin B and clotrimazole were both able to inhibit the response to a variety of stimuli of lymphocytes in an artificial medium.[48] The amphoteric polyenes, amphotericin B and nystatin had an adjuvant effect on the in vitro antibody response of mouse spleen cells to heterologous erythrocytes, whereas the non-amphoteric filipin suppressed the response.[49] In mice, hydroxystilbamidine isothionate suppressed both the primary and secondary immunological responses to bovine serum albumin.[50] Griseofulvin added to mammalian cells in vitro produced aggregation of chromosomal sets and may provide a useful adjunct for linkage and chromosomal assignment studies.[51] Cats given 500-1000 mg. griseofulvin orally every week in the first half of **pregnancy** produced kittens with multiple congenital abnormalities.

Nystatin and mycoheptyne were given to pregnant Wistar rats. Both had a slight abortive effect when used during the whole pregnancy. No fetal defects were found.[53]

Amphotericin B remained the principal antifungal agent for the treatment of systemic fungal infections but the methyl ester (AME) which is water soluble was further studied. As a solid and in solution at pH.6 it had a similar stability to amphotericin B.[54] In acid solutions, however, there was a 90% loss in activity over 21 days. In vitro susceptibility testing showed the spectrum of activity to be the same although AME had a lower level of activity.[55] Similar results were obtained in experimental histoplasma, blastomycoses, cryptococcal and candidial infections of mice.[56] In mice and dogs, AME was 1/8 - 1/25 less toxic than amphotericin B.[57] Following the intravenous injection of ^{14}C methyl ester, 51% was recovered in the first four days in the feces; this fell to 15% after an intraperitoneal injection. No detectable parent compound was found in the urine.[58]

The combination of amphotericin B with other agents continued to receive attention. The action of amphotericin B on Coccidiodes immitis in vitro was greatly enhanced by the addition of polymyxin B. Polymyxin C (Colistin) which is non-cationic, was inactive in this respect.[59]

Variable responses to the in vitro testing of 5-FC and amphotericin B against C. neoformans, C. albicans and other Candida spp. were obtained. Synergy, when observed, was mainly in partially 5-FC resistant strains.[60] Within therapeutically achievable levels, this combination could result in

a candicidal as opposed to a static effect. [61] No advantage was established against C. neoformans in experimental infections of mice. [62] Patients with disseminated cryptococcosis who received the combination had reduced periods of hospitalization and the toxic effects of amphotericin B were reduced. [63] As well as potentiating the action of antifungal agents, amphotericin B enhanced the activity of 1, 3-Bis (2 chloroethyl)-1-nitrosurea against transplanted A2K leukemia. [64] Another polyene, pentamycin, potentiated the action of both bleomycin and ∝-amanitin against transformed animal cells in tissue culture. [65]

Mathematical equations of the pharmakokinetics of 5-FC in patients with renal impairment were discussed. [66] 5-FC with protein binding of less than 5% had similar clearance characteristics to creatinine in patients undergoing hemodialyis, whereas only 3-15% of amphotericin B, with strong binding to protein, was cleared. [67] The clearance of miconazole from serum was not affected by the patient's renal function. [68] The absorption of griseofulvin from the gut was improved if it was micronised in an emulsion of corn oil and water. [69] Griseofulvin may have two mechanisms for stimulating 5-aminolevulinate synthetase production - a non specific effect which has also been obtained with some other lipid soluble drugs and a more specific interference with the feed back of heme. [70] Hamycin reduced serum cholesterol in rats by 50% when given orally or intraperitoneally. [71]

Clotrimazole was active against Naegleria fowleri in vitro, but failed to protect infected mice. [72] Both clotrimazole and miconazole were effective in protecting mice against inoculated C.immitis. [73, 74].

The rabbit was used as an experimental model to study oculomycoses. Severe infections of the cornea with C. albicans responded to oral and especially combined oral and topical treatment with 5-FC. Topical treatment alone was only effective in mild infections. [75] Corneal infection with Fumigatus keratitis was unaffected by clotrimazole used either orally or topically. [76] Amphotericin B injected into the vitreous humour led to local inflammation, cataracts and retinal detachment even at levels unlikely to be effective against fungi. [77]

Clinical Studies - In a retrospective study of 51 cases of North American blastomycosis, 11 were considered to have involvement of the genito-urinary tract. Treatment with amphotericin B reduced the mortality rate from 90 to 10% with a 10-15% relapse rate. [78] Six children aged between 1 and 42 months with severe active histoplasmosis were treated with amphotericin B for 7-11 days. In the follow-up period, only one child relapsed. [79] A further two cases of mycotic keratitis due to Fusarium responded to a 5% suspension of pimaricin. [80]

5-FC orally was used successfully in the management of a boy with endocarditis caused by Rhodotorula pilimanae. [81] A man with an aortic valve homograft developed a T. glabrata endocarditis which failed to respond to amphotericin B therapy. The disease process was halted by 5-FC and although the damaged valve required replacement, the patient

made a satisfactory recovery. [82]

Miconazole as a 2% cream was effective in the treatment of skin infections due to T.rubrum and T. mentagrophytes. [83] Compared with chlordantoin in vaginal candidiasis the overall cure rate was significantly better with miconazole. [84] A vaginal infection with T.glabrata failed to respond and the strain was resistant on in vitro testing. [85] For systemic fungal infections miconazole gave better therapeutic levels when administered intravenously rather than orally. [86] Four patients with generalised coccidiodomycosis were given intravenous miconazole. Improvement was seen in two patients with acute pulmonary disease. [87] A further nine patients who had failed to respond to amphotericin B received miconazole; 7 improved. Side effects included phlebitis, dizziness, nausea and macula papular rashes, and all were reversible on stopping treatment. [88]

Griseofulvin was applied topically in an alcoholic solution to an experimental infection with T. mentagrophytes but was of no value when the infection was established. [89] In a 2% micronised cream, it compared favorably with 1% tolnaftate cream in natural dermatophytic infections. [90]

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Chapter 12. Antineoplastic Agents

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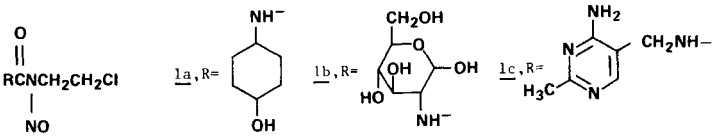
Introduction - Several important review articles dealing with the pharmacology and clinical activity of both new and established antitumor drugs have appeared recently. An especially useful and comprehensive two-part review[1] of all major antineoplastic and immunosuppressive agents has been published. Part I treats test systems, the rational design of alkylating agents, antifols and nucleosides, cell cycle kinetics, drug transport, metabolism, toxicity and resistance, combination chemotherapy, immunotherapy and the effect of ionizing radiation. Part II has 50 chapters concerning the biochemistry, pharmacology and mechanism of action for specific classes of antitumor agents. The current status of platinum, rhodium and Group III metal complexes as antitumor agents has been reviewed[2], as has the *in vivo* and *in vitro* antitumor activity of 1500 quinones[3]. The National Cancer Institute (N.C.I.) has available[4] two compilations listing structures and murine antitumor activity for (a) 47 drugs with clinical activity and (b) 35 compounds of unique structure under development by the N.C.I. The newest edition of "Goodman and Gilman"[5] has an updated chapter on antineoplastic agents. Recent advances in the pharmacology of clinically effective agents [methotrexate (MTX), 5-fluorouracil (5-FU), arabinosyl cytosine (ara-C), adriamycin, bleomycin, nitrosoureas and cyclophosphamide][6], the current status of clinical immunotherapy[7] (BCG, MER-BCG, *Corynebacterium parvum*, poly I-C and levamisole) and the techniques of pharmacokinetic modeling of drugs[8] have also been reviewed. A study of animal brain tumor models has compared therapeutic effects of many of the major solid tumor agents[9]. A comprehensive review of all aspects of the important drug, adriamycin,[10] and a discussion of the clinical utility of high dose methotrexate therapy[11] has been reported.

Drugs which have appeared promising in pre-clinical studies often disappear from the literature during the initial clinical evaluation period, which can be rather lengthy. The N.C.I. has available two reports which discuss (a) the current status of the early (Phase I) clinical trials[12] of cyclocytidine, fluorocyclocytidine, piperazinedione, inosine dialdehyde, Baker's antifol, cytembena, asaley, ftorafur, thalicarpine, gallium nitrate, D-tetrandrine, S-trityl L-cysteine, MER-BCG, IV CCNU, dianhydrogalactitol and pyrazofurin and (b) clinical data on investigational drugs which have been in clinical trial for a more extended period[13] [nitrosoureas, hexamethylmelamine, cis-dichlorodiammineplatinum, chromomycin A_3, isophosphamide, 5-azacytidine, VM-26, VP-16, Yoshi 159 and ICRF 159].

Alkylating Agents - The nitrosoureas are still under active study. Much of the research activity in nitrosoureas has turned to the pharmacology, clinical activity and mechanism of action of these drugs. The cis and trans-4-hydroxy derivatives (1a) which had been shown to be human metabolites of CCNU, were synthesized, evaluated and suggested to be the active forms of CCNU[14]. Using the strategy successfully employed earlier with the aliphatic analogs, the Southern Research Institute group replaced the methyl group of the fermentation product, streptozotocin, by a β-chloroethyl function to produce

chlorozotocin[15], a water-soluble nitrosourea carbohydrate (1b), which has much greater L1210 leukemia activity than the natural product. It has significantly lower bone marrow toxicity than the previously prepared aliphatic analogs. A water-soluble pyrimidine nitrosourea analog (1c) was reported to have higher L1210 activity than the best of the aliphatic derivatives[16]. Certain psychotropic drugs such as chlorpromazine and chlordiazepoxide enhanced the therapeutic effect of BCNU against L1210 leukemia[17]. Somewhat modified mechanisms for the decomposition of nitrosoureas in solution were proposed[18,19].

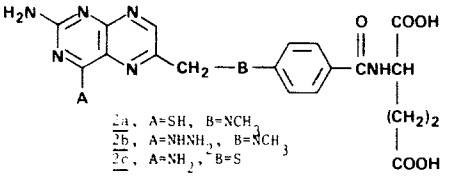


The 4-trifluoromethyl analog of cyclophosphamide was synthesized in a successful attempt to increase the toxicity of the aldehydic degradation product[20]. Cyclophosphamide was reported to cause a great increase in pulmonary metastases in a mouse tumor model[21]. Cyclophosphamide isomers such as isophosphamide, however, had a much reduced effect. Hydantoins containing the nitrogen mustard function were prepared as CNS antitumor agents and were curative in the ependymoblastoma mouse brain tumor model[22]. Tetraacetylglucosamine mustard[23] gave increase in life span (ILS) values of 100% and 200% in L1210 and P388 leukemia, respectively. The presence of β-haloethyl and secondary amine groups were found to be important in the conversion of an inactive mustard to an L1210-active degradation product[24]. Chlorambucil, noncovalently bound to antimelanoma globulins, formed a complex which was active against human metastatic skin melanomas[25].

Based on a proposed mechanism of action for AB-132, an N-hydroxy analog, bis(aziridinyl)phosphinyl-N-hydroxyurethane (AB-182), was prepared which was much more active than AB-132 as an X-radiation potentiator[26]. Mass spectral studies showed that the active metabolite of 1-(1-aziridinyl)-2,4-dinitrobenzene (CB-1837) in WA 256 was the 2-amino compound[27]. Most of the naphthoquinones prepared as bioreductive alkylating agents[28] were active against Sarcoma 180 ascites *in vivo* with the most active compounds having ILS values > 100%. A possible mechanism for the antitumor action of an imidazole carboxamide triazene mustard (NSC 82196) was proposed which involves the generation of a carbonium ion[29].

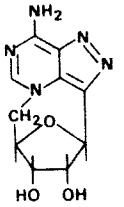
Folic Acid Antagonists - The quinazoline analog of isofolic acid and its 4-amino derivative were inactive in the L1210 model but were dihydrofolate reductase (DFR) inhibitors[30]. Marginal L1210 activity was observed when a thiolactone or an aspartic acid ester replaced glutamic acid in MTX derivatives[31]. The 4-thio (2a) and 4-hydrazino (2b) analogs of MTX were synthesized[32]. While they were found to be L1210-inactive and over 1000 times less inhibitory to pigeon liver DFR than the parent compound, the 4-thio compound was an excellent inhibitor of the growth of *Streptococcus faecium*. The 10-thio analogs of folic acid[33,34] and aminopterin[34] (2c) were prepared and found to be active as *S.faecium*, *L.casei* and DFR inhibitors. Several esters in this series inhibited a MTX resistant strain of *S.faecium*. Better

cell penetration properties were postulated as the reason for the activity[34].



2a, A=SH, B=NCH$_3$
2b, A=NHNH$_2$, B=NCH$_3$
2c, A=NH$_2$, B=S

Nucleosides - Cordycepin (3'-deoxyadenosine) was shown to inhibit murine *sarcoma* virus production induced by iododeoxyuridine[35]. The syn (3) and anti (4) forms of formycin cyclonucleoside (anhydroformycin) were synthesized and found to be inactive against the Ehrlich and L1210 tumor models[36]. The syn compound was resistant to two adenosine deaminases while both formycin and the anti anhydro derivative were deaminated. Nucleosides of 2-azaadenine and 2-azahypoxanthine were synthesized for in vitro



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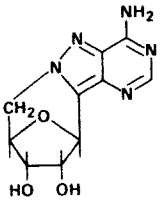

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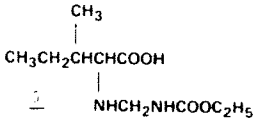
evaluation. While the 2-azaadenine derivatives were phosphorylated, 2-azahypoxanthine nucleosides were converted to the parent base which was cytotoxic[37]. The 5'O-formate ester of arabinosyl adenine was prepared as a pro-drug and found to be 60 times as water-soluble as the parent[38]. Esters of ara-C were synthesized as depot forms of the parent. The L1210 activity was correlated with water solubility and mixed esterase hydrolysis rates[39]. Tetrahydrouridine, a pyrimidine nucleoside deaminase inhibitor, was found to increase the L1210 activity of orally administered 5-azacytidine to the level obtained when 5-azacytidine alone was administered intraperitoneally[40]. Since 3-deazauridine had been found to have antitumor activity, the 1,3-dideaza analog was prepared[41]. It was active against herpes simplex virus but inactive against L1210 cells in vitro.

Amino Acids and Amino Acid Derivatives - The 5 most active amino acids[42] from a group of 350 evaluated in four tumor systems[43] were studied further in solid and ascites S-180. N-Ethylcarbaminomethyl-L-isoleucine (5) appeared to have the best overall activity. L-Arginine inhibited the growth of carcinogen induced mammary tumors in rats[44] and N-[p-(fluorosulfonyl)-benzyl] derivatives of asparagine and glutamine produced a moderate inhibition of the enzyme asparagine synthetase[45].



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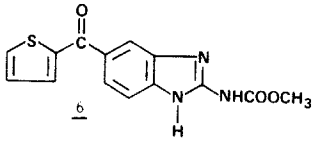

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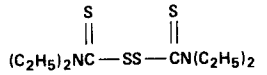
Synthetic Agents - Methyl [5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl] carbamate (R17934, NSC 238159, 6) is active against the leukemia L1210 (ILS 82%), P388 (195%), IP B16 melanoma (108%) and the IM Lewis lung (36%) tumor systems[46]. It is a mitosis inhibitor which is synergistic with ara-C against leukemia L1210. Hydantoins substituted in the 3-position with purines were active at 10^{-4}M concentrations against L1210 cells *in vitro*[47], while nitrogen analogs of mycophenolic acid[48] were inactive *in vivo* (L1210, WA 256). 2-Formyl-4-(m-amino)phenylpyridine thiosemicarbazone was very active in the Sarcoma 180 ascites system (ILS 162%)[49]. The *ortho* and *para* amino isomers were inactive while the unsubstituted compound gave an ILS of 50%.

A new class of platinum-containing antitumor agents, the "platinum blues" contain Pt complexed with nitrogen heterocycles such as uracil. While the structures of most of these derivatives are uncertain, several materials are more active against the Sarcoma 180 ascites system and cause fewer kidney lesions than *cis*-dichlorodiammineplatinum[50]. Nine N-demethylated derivatives of hexamethylmelamine were tested in the S-180 and Lewis lung models. Results suggest that activity is related to the presence of, rather than the number of, methyl groups in the molecule[51]. Disulfiram[52] (7) and DDT[53] protected mice and rats against carcinogen-induced tumors. An aromatic analog of retinoic acid (Vitamin A acid) known as Ro 11-1430 (8) was synthesized in a successful attempt to reduce the side effects of the parent drug. Ro 11-1430 was active against murine epithelial tumors and had a ten-fold better therapeutic index than the parent[54]. Analogs of thalidomide[55] were as active as mitomycin C against the Ehrlich ascites



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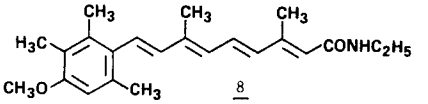

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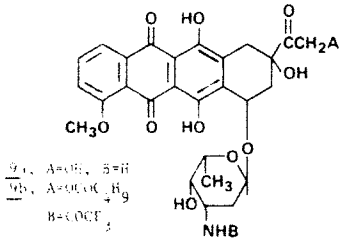
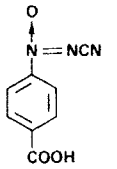
tumor and simple mercaptoalkylamines, e.g., cysteamine, inhibited solid tumor growth or caused tumor necrosis[56]. The unsaturated aldehyde, 4-hydroxy-2-penten-1-al, inhibited the *in vitro* uptake of ^{3}H-thymidine in Ehrlich ascites[57,58] and S-180[58] cells. Pyran copolymer was found to be useful as an adjuvant interferon inducer with a remission inducing agent such as a nitrosourea[59]. The compound also prophylactically inhibited Friend leukemia virus *in vivo* when administered IP. The disease was enhanced when the compound was administered intravenously[60].

Fermentation Products - Adriamycin (9a) and its analogs continued to be the objects of intensive research due to the promising clinical activity of adriamycin in the treatment of solid tumors[10]. N-Trifluoroacetyladriamycin-14-valerate (AD32, 9b) was found to have much greater murine leukemia L1210 and P388 activity than adriamycin[61]. In addition to giving multiple cures in these tumor systems, AD32 displayed a lack of delayed toxicity. Adriamycin and daunorubicin analogs with 4'-epimeric hydroxyl groups had activity comparable to the parent drugs but not the toxicity to cultured heart cells shown by adriamycin[62]. The Russian antitumor antibiotic, carminomycin, was shown to be 4-O-demethyl daunorubicin by X-ray crystallography[63]. A comparison of daunorubicin and its DNA complex gave identical

leukemia L1210 ILS values[64]. The antitumor properties of the DNA complex, however, were shown to be a function of numerous factors[65] including the nature of the DNA and the method of preparation of the complex[66]. The cellular pharmacodynamics of daunorubicin and adriamycin were explored. On the basis of in vitro, murine in vivo and human clinical response data, it





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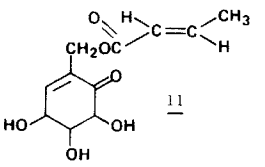

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was hypothesized that daunorubicin penetrates the cell and the carbonyl group at the 13-position is enzymatically reduced to give daunorubicinol[67]. This metabolite may play an important role in the observed therapeutic response of daunorubicin. Significantly less carbonyl reduction was observed in adriamycin experiments. Eight daunorubicin metabolites were identified in human urine[68]. These studies show that the human metabolism of daunorubicin involved carbonyl reduction, reductive glycosidic cleavage, O-demethylation, O-sulfation and O-glucuronidation. Aclacinomycin A and B are trisaccharide anthracycline antibiotics similar to the cinerubins[69]. One of these compounds showed an ILS of 200% in leukemia L1210 without the hamster ECG change produced by adriamycin.

Three 7-substituted actinomycin D derivatives (NO_2, NH_2, OH), synthesized from the natural product, had approximately the same antitumor activity (L1210, P388, Ridgway) as the parent[70]. Replacement of the 2-amino group in the parent with hydrogen or 3-hydroxypropylamino groups led to the conclusion that the 2-amino group is important but not essential for antitumor activity[71]. Ergot derivatives were prolactin inhibitors which were active against diethylstilbesterol initiated renal tumors[72] and dimethylbenz[a]-anthracene induced mammary carcinomas[73]. 6-Cyano-6-norlysergic acid derivatives, prepared as potential CNS antitumor agents, were L1210 inactive[74].

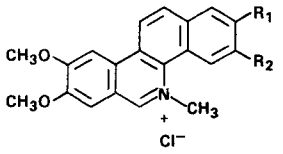
Calvatic acid (10), a mushroom fermentation product, prolonged survival time 53% in L1210 leukemia[75]. An L1210-active isoxazole acetic acid antibiotic (U-43,795) was isolated from S.sviceus[76]. This compound is the 4-hydroxy analog of a previously isolated antibiotic and is a powerful inhibitor of many mammalian and bacterial amidotransferase enzymes utilizing L-glutamine[77]. A material isolated from S.griseosporeus, 2-crotonyloxymethyl-(4R,5R,6R)-4,5,6-trihydroxycyclohex-2-enone (11) reacts with sulfhydryl groups and is active against the solid Ehrlich tumor and leukemia L1210 (ILS 77%)[78].

Plant Products - Maytansine is an ansa macrolide with in vivo leukemia P388 activity at microgram per kilogram doses. It was cross-resistant with vincristine in P388 leukemia, and by using flow microfluorometric techniques, was determined to arrest mitosis in metaphase in a manner analagous to the vinca alkaloids[79]. Two new maytansinoids were isolated from Putterlicka verrucosa Szyszyl. Maytanacine (P388 ILS 130%) is the first analog without

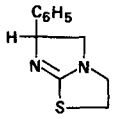
an amino acid residue at C-3[80]. Tritiated vincristine was used to determine the uptake, binding and metabolism of that drug in L1210 and P388 cells[81]. Drug resistance is due to impaired accumulation and binding in the cell.

Structural requirements for nitidine[82,83] (12a) and fagaronine[83] (12b) antitumor activity were determined. A kinetic study of ring hydration, opening and reclosure in the coralyne series was carried out[84]. A modified synthetic scheme allowed the synthesis of 8,9-dialkoxy ellipticine analogs. While these compounds were not active, eleven other derivatives had some activity against solid S-180 and Ehrlich carcinoma[85]. Amygdalin MF (laetrile) was inactive when tested alone or in combination with β-glucosidase in the L1210, P388, B16 melanoma, WA 256, Lewis lung and Ridgway osteogenic sarcoma tumor systems[86,87]. N-Acetyl-methylthiocolchicine was as active as colchicine against L1210 cells *in vitro*[88].

Gossypol, the yellow pigment from cottonseed, was isolated from *Montezuma speciosissima (Malvaceae)* and gave an ILS of 50% in the leukemia P388 system[89]. Bruceantin was isolated from an Ethiopian plant source. This compound was active both *in vivo* (L1210, P388, Lewis lung, B16, WA 256) and *in vitro* (KB)[90]. Crassin acetate, isolated from several species of Caribbean marine organisms, was KB active with marginal (ILS 30%) P388 leukemia activity[91]. Oral administration of either Δ^8 or Δ^9-tetrahydrocannabinol gave ILS values of 25-36% in the Lewis lung tumor model[92].



12a, $R_1 = R_2 = OCH_2O$
12b, $R_1 = OH$, $R_2 = OCH_3$



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Immunotherapy - Levamisole (13), a non-specific stimulant of the immune system, and its racemic isomer tetramisole were inactive against a standard series of murine tumors (L1210, P388, B16, Lewis lung and Madison 109)[93]. When used in combination with a remission-inducing agent such as BCNU, levamisole gave an augmented therapeutic response in the MCAS-10 tumor system. This was attributed to immunostimulant action[94]. CNS active drugs (picrotoxin, apomorphine, ephedrine and strychnine) cured rats after surgical removal of methylcholanthrene induced tumors[95]. Picrotoxin was the most effective agent but it had a very narrow therapeutic range.

Corynebacterium parvum, BCG and MER-BCG continued to be the subject of many immune response studies in tumor systems[7]. A single injection of *Corynebacterium granulosum* caused complete and lasting rejection of a carcinogen-induced mouse tumor[96]. Bacterial core lipopolysaccharides from *S.minnesota R* mutants showed a 100% ILS in the Ehrlich ascites tumor model[97]. The lipid portion was crucial since the oligosaccharide from the material was inactive.

Miscellaneous Agents and Techniques - Hematoporphyrin was preferentially retained in WA 256 and carcinogen induced tumor tissue. The tumor was

destroyed when the treated animals were irradiated with light of wavelengths greater than 600nm[98] and cures were obtained in both tumor systems. As much as 0.1% of the incident light was calculated to penetrate 7 cm into the tumor tissue. Singlet oxygen was proposed as the cytostatic agent. Hematoporphyrin destroyed glioma cells in the presence of light[99]. Triphenylmethane dyes such as pyrocatechol violet were shown to be potent inhibitors of reverse transcriptase from Rauscher leukemia virus[100].

Nuclear magnetic resonance (nmr) spectroscopy was used to categorize a large number of normal and neoplastic tissues from humans[101] and animals[102]. The differences found in proton relaxation times (T_1 and T_2) were attributed to increased water content in tumor tissue. While several research groups judged the nmr method less promising for detecting cancerous tissue than originally thought[101,102], other workers were more optimistic[103]. Electron spin resonance techniques were used to study the blood, spleen and liver of mice with myeloid leukemia [104]. Changes in the concentration of iron and copper complexes in all three tissues appeared to be related and were attributed to iron transport properties. Hyperbaric hydrogen was shown to cause tumor regression in the murine squamous cell carcinoma system[105].

Structure-Activity Studies - The role of structure-activity studies in the design of antitumor agents was outlined and emphasis was placed on the importance of the partition coefficient and drug transport properties. The concept of a double pro-drug liberating the active material by a "cascade latentiation" process was also described[106]. An extensive study in the 4'-(acridin-9-ylamino) methanesulfonanilide series showed a parabolic relationship between lipophilic character and leukemia L1210 activity[107]. A modified Free-Wilson approach was successfully applied to a quantitative correlation between DFR inhibition and the structures of 105 2,4-diamino-5-(3,4-dichlorophenyl)-6-substituted pyrimidines[108]. A qualitative correlation of *in vivo* murine intracerebral Glioma 26 inhibition was made with 7 imidazolecarboxamide triazene derivatives[109]. This study concluded that for an optimal response, drugs with greater lipophilic character are required for the treatment of solid tumors than leukemias. Pattern recognition techniques were used to predict the activity of 24 test compounds in the ependymoblastoma intracerebral mouse tumor system. It was suggested that this technique may be useful in generating new "lead" compounds[110].

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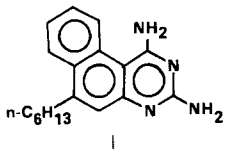
Chapter 13. Antiparasitic Agents

Edgar J. Martin, Food and Drug Administration, Rockville, Md.

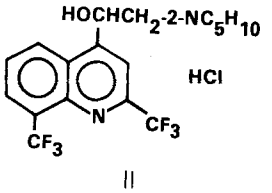
Introduction - Reviews appeared on the circadian rhythms of parasites and their interrelations with those of their hosts;[1] on immunity against certain parasitic worms;[2] and on fused pyrimidines as folate antagonists.[3] A monograph on insect hormones has appeared.[4] The need for screening all drugs for mutagenic potential was emphasized.[5]

Antimalarials - Reviews appeared on recent developments in malaria and filaria chemotherapy;[6] on the status of malaria immunity;[7] on the U.S. Army development of antimalarials;[8] and on antimetabolites of coenzyme Q as antimalarials.[9]

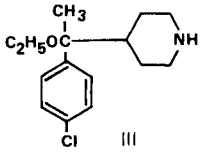
The squirrel monkey, Saimiri sciureus, was found to be susceptible to Plasmodium vivax, and suitable for antimalarial drug testing.[10] A new fluorometric method has been used to determine amodiaquin in serum, plasma and red cells.[11] The use of drug mixtures in rodent malaria may prevent formation of drug resistance against one of the components but not against the others.[12] In a series of arylaminoureas, regression lines developed from potency data of a few analogs suggest theoretical predictability of activity of other analogs; the system was tested on P. vinckei, P. berghei, and P. knowlesi.[13] The following compounds were found active against P. berghei: Hydrophobic analogs of diaminoquinazoline acting as folic acid antagonists;[14] the benzoquinazoline I;[15] the quinoline II;[16] the chlorophenyl analog III;[17]



I

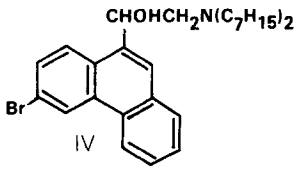


II

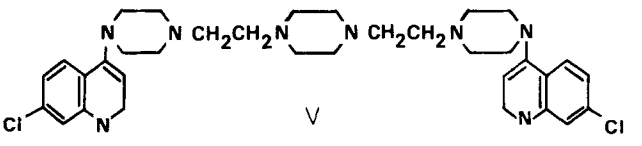


III

Resolution of compound IV was achieved through its tartrate salt; the le-



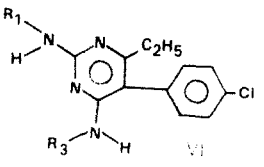
IV



V

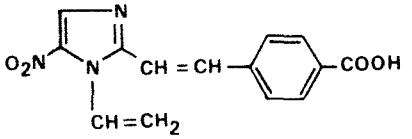
vorotatory isomer was found active.[18] Some 9-phenanthrene methanols which were simultaneously substituted at the 2, 7 and 10 position showed activity.[19] The tris-piperazine bis [7-chloroquinoline] analog M 1020 V

was found effective in clinical trials against P. vivax and P. falciparum.[20] Some derivatives of phenyl chloromethyl sulfone showed weak antimalarial activity.[21] The pyrimethamine (P) derivative VI which has R_1 and R_3 = $(C_3H_7)_2CH-CO-$ was found more effective and less toxic than (P) as tested on P. berghei in mice, and as effective as (P) against P. gallinaceum in chicken.[22] Certain methotrexate analogs were found active against P. gallinaceum in chicks but not against P. berghei in mice.[23] Various sulfonamides were reacted with silver nitrate. Only the sulfadiazine product was effective against P. berghei in mice. It was of acceptable toxicity.[24] Antimalarial lincomycin analogs have been reviewed. The most active ones were those modified at the C7 position of the sugar moiety.[25]

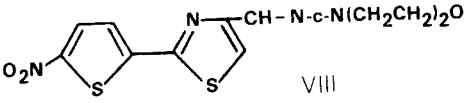


VI

Antitrypanosomals and Antileishmanials - Reviews appeared on the chemotherapy of leishmaniasis[26] and on metabolic inhibitors and antitumor antibiotics which are trypanocidal.[27] The screening of 65 diaminoquinazolines showed that the antifolate effects against bacteria and trypanosomes varied independently.[28] The activity of thiosemicarbazones against Trypanosoma cruzi is eliminated if the sulfur is replaced by oxygen.[29] The activity of compound VII against T. rhodesiense is reduced if the carboxyl group is replaced by a basic function.[30] Among thiazole derivatives, VIII was



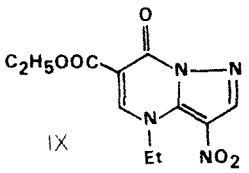
VII



VIII

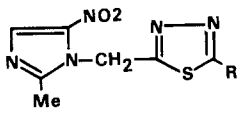
the most active against T. cruzi and T. rhodesiense; insertion of an ethylene bridge between the heterocyclic rings abolished the activity.[31]

Antitrichomonal Agents - In a series of pyrazolo-pyrimidines, the compound IX was the only one active in vitro against Trichomonas foetus. The removal of the 4-ethyl group abolished the activity.[32] Some of 71 tested nitroheterocycle analogs had in vivo activity in mice against T. vaginalis comparable to that of metronidazole.[33] In a series of 2-nitrobenzofuran-alcohols, the antitrichomonal and antiamebic activity was found to be reduced by etherification and esterification of the hydroxyl group and also by bromination of the carbocyclic ring.[34] Triacylmethane derivatives were synthesized and some of them were found active against T. vaginalis in vitro.[35] The antibiotic Viridenomycin, isolated from a Streptomyces viridochromogenes strain was found to be active against T. vaginalis.[36]

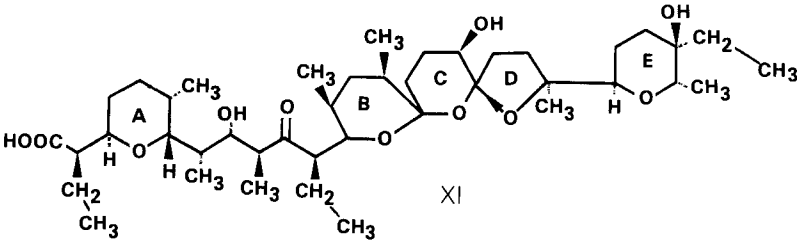


IX

Anticoccidia and Antitoxoplasma Agents - A review appeared on anticoccidials.[37] The derivatives R=H or NH_2 or NHCOMe of the parent compound X were active against various Eimeria species.[38] Eimeria tenella strains which were resistant to a variety of drugs showed no cross resistance to Lasalocid in chicks.[39] In deoxyisopyridoxal and norisopyridoxal a-CHO group in the 5 position was necessary for anti-coccidial activity.[40] Compounds which were active against E. tenella in chicks were obtained when 2 substituted hydrazines were linked either directly or through an allyl group; the toxicity of dithiolcarbamoyl hydrazines was reduced in metal chelates.[41] The polyether antibiotic, Salomycin (Kaken) XI, was extracted



X

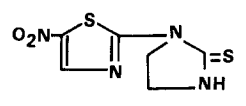


XI

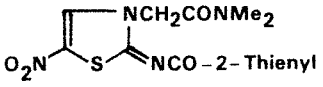
from cultures of Streptomyces albus; it was effective against coccidial infections in poultry.[42] Homologs of Lasalocid A were isolated and found active against coccidia in chicks.[43] Certain synthetic polyribonucleotides protect mice against toxoplasma; this effect may be due to nonspecific enhancement of immune mechanisms.[44]

Amebicides - A review appeared on the evaluation of amebicidals.[45] Some 200 nitroanilines were screened for biologic activity; several dinitrobenzenes with 2 identical basic groups were found active against amebiasis in hamsters.[46] 5-Nitrothiazole-2-ylsemicarbazides were synthesized and some of them were found active against Entameba histolytica in hamsters and T. vaginalis in mice.[47] The aminoglycoside antibiotic G-418 (Schering) was produced from micromonospora, and was found active against E. histolytica, Hymenolepis nana, Syphacia obvelata, and Taenia in dogs and cats. It was less effective than metronidazole against T. vaginalis in mice.[48] In a series of 2,2'-bisimidazole derivatives a 5-nitro group was found necessary for activity against E. histolytica, T. vaginalis, Giardia muris. An added amino group enhanced the activity.[49]

Antischistosomal Agents - In a series of nitrothiazole derivatives the compound 26354 R.P., XII, had good activity against Schistosoma mansoni in mice and Macacca mulatta.[50] Among 23 nitrothiazolines and 22 nitrothiazole analogs the compound XIII showed good anti-S. mansoni activity in mice and rhesus monkeys.[51] Replacement of the nitrothiazole in certain nitrofurylic acid amides and substitution of nitrothiazole by nitrofuran in

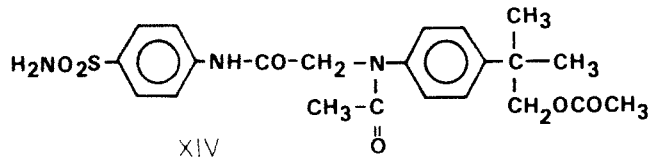


XII



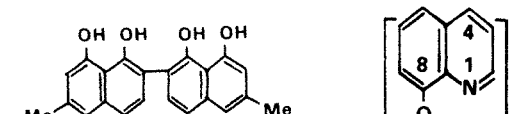
XIII

niridazole and some of its analogs caused loss of antischistosomal activity.[52] Naphthylenediamine salts were found in vitro active against S. mansoni.[53] Diaminodiphenylsulfone (DDS) was found to interfere with the egg laying of S. mansoni in mice, hamsters, and Cebus monkeys, but was not effective in man.[54] The metabolite XIV of BAY d 9778 showed slight antischistosomal activity in Cebus monkeys.[55] Quinine and some of its derivatives caused changes in egg elimination and hepatic shift of S. mansoni in mice and hamsters.[56] Teratogenicity and/or embryotoxicity of hycanthone was observed in mice and rabbits.[57]



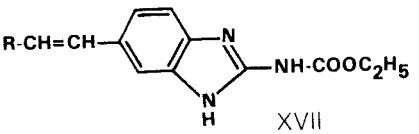
XIV

Other Anthelmintics - In vitro cultures of the gastro-intestinal helminth Cooperia punctata were used for evaluating the activity of 28 anthelmintics.[58] The diosporyl derivative XV was active against Necator americanus in hamsters.[59] Some antimony complexes of 8-hydroxyquinolines, e.g., XVI were active against Hymenolepis nana.[60] In a series of benzimidazoles the analog R = $CH_3O.C_6H_4$ of XVII was found active against H. nana in mice.[61] In a series of thiocyanatobenzothiazoles there were active as well as inactive analogs against Ascaris suum and/or H. nana; no structure-activity relationship was established.[62] The compound XVII was found to be a broad-spectrum anthelmintic.[63] The naturally occurring bithienyl nematocide XIX was synthesized.[64] Various 2-ethyl phenol analogs were found to be active against A. suum.[65] Activity of piperazineguanidine XX analogs against Syphacia obvelata was tested in mice; the XXa derivative was found 5 times more



XV

XVI



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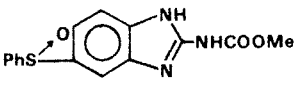

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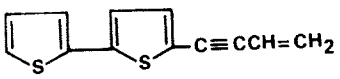

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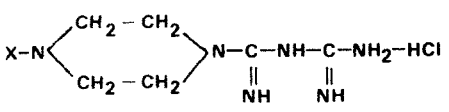

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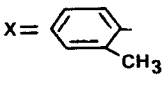

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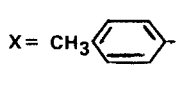

XXb

active than the XXb derivative.[66] The antibiotic Destomycin C showed similar anthelmitic activities as Destomycin A and B.[67] The importance of piperazine side chains in anthelmitics was studied. Some N-methyl-piperazine substituted benzimidazoles are active against Litomosoides carinii in cotton rats.[68] In a series of 61 analogs of diethylcarbamozine four compounds had similar activity as the mother substance against L. carinii in cotton rats. The structures of these compounds did not suggest any pattern of structure/efficacy predictability.[69]

Pneumocystis carinii - The combination of antifolates trimethoprim + sulfamethoxazole (Bactrim) was reported to be effective in the treatment of Pneumocystis carinii pneumonitis in immune suppressed subjects.[70] The use of a similar antifolate combination, pyrimethamine + sulfadoxine has been previously reported for the use in the infantile form of pneumocystosis.[71]

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Chapter 14. Antiviral Agents

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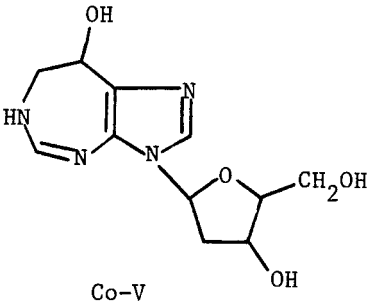
Introduction - Several reviews on the present status of antiviral chemotherapy have been published recently[1,2,3]. In addition, reviews on the treatment of virus diseases have appeared, with that of Juel-Jensen[4] covering the field in general and others concerned with the treatment of certain virus diseases such as herpes zoster[5], genital herpes[6] virus infections of the outer eye[7] and influenza A infections[8]. A symposium, "Antivirals with Clinical Potential", which updated all advances in this area, was held at Stanford University, August 26-29, 1975. Of interest to those engaged in antiviral chemotherapy is an article concerned with the strategy for discovering and developing chemotherapeutic drugs[9] and one concerned with the use of specific inhibitors of viral replication as potential prophylactic antiviral agents[10].

During the past year much of the virus chemotherapy research was directed at herpes and other DNA viruses and this included clinical and laboratory studies on known compounds in order to ascertain their spectrum of clinical efficacy.

Adenine Arabinoside (Ara-A, Vidarabine) - Extensive clinical trials have been carried out with ara-A on a variety of virus infections and by different treatment routes. Topical treatment of herpetic keratitis with 3% ointment was reported effective and a good alternate to IDUR therapy[11,12]. Genital herpes has proven more refractory and double-blind studies showed no differences in activity between topical placebo and topical drug treatment[13,14]. Parenteral (IV) administration of ara-A was reported to be curative and well tolerated in the early treatment of severe disseminated herpes infections in infants and adults[15,16]. Poor results were obtained with ara-A treatment of confirmed herpes simplex encephalitis, and the authors concluded that more effective agents are needed[17]. These results contrast with the successful treatment of herpes simplex encephalitis by ara-A in mice[18]. Clinical studies with IV ara-A treatment of disseminated herpes zoster infections suggested a beneficial effect, but overall activity was difficult to assess due to the high rate of spontaneous recovery[19,20]. A possible beneficial effect was also reported for IV ara-A treatment of varicella zoster infections in immunologically competent patients[21]. Dramatic improvement was reported in two men with severe chicken pox treated IV with ara-A[22], and the authors recommended its use for these infections. Ara-A was found to be completely ineffective for treatment of variola major infections as shown by a double-blind study in 18 patients[23]. A problem with ara-A is its rapid enzymatic conversion to the less active arahypoxanthine (ara-Hx) by adenosine deaminase. A new enzyme inhibitor (Co-Vidarabine, Co-V) has been reported to potentiate the activity of ara-A in tissue culture[24,25] and to reduce the deamination of ara-A in rabbit eyes[26] and in monkeys[27] suggesting possible clinical application of combined ara-A - Co-V. The synergistic effect reported for

the treatment of herpes simplex encephalitis infections of mice with ara-A and humoral antibodies suggests another potential combination for clinical application[28].



Co-V

Three 5'-monophosphate ester analogs of ara-A were as active as ara-A against several viruses in tissue culture[29]. One of these, arahypoxanthine-5'-monophosphate (ara-HxMP), was found to be well tolerated and active against experimental herpes virus infections in mice[30] and rabbits[31], against equine abortion-induced hepatitis in hamsters[32] and against DNA virus-induced encephalitis of mice[33]. For IV dosing, the greater water solubility of ara-HxMP over that of ara-A was a marked advantage.

Cytosine Arabinoside (Cytarabine, Ara-C) - Recent clinical trials with ara-C have provided variable results. Although SC administration of ara-C provided antivirally effective blood concentrations, this treatment was ineffective against localized herpes zoster infections[34,35]. A double-blind study of ara-C in variola major patients demonstrated no activity and possibly a deleterious effect[36]. In contrast to these negative results, IV administration of ara-C was reported to be effective for the treatment of herpes zoster, herpes simplex and cytomegalovirus infections in renal allograft recipients[37] and recommended for use in such patients.

Idoxuridine (IDUR, IDU - Iododeoxuridine) - IDUR has been available for many years for treatment of herpetic keratitis with clinical trials in other areas continuing in order to expand its usefulness. In children with cancer, cutaneous varicella and varicella-zoster pneumonia (but not localized herpes zoster) infections were reported to respond to IDUR therapy[38]. Although there have been some reports of efficacy, in general, IDUR treatment of herpes encephalitis has been unsuccessful. Two recent studies showed no activity and severe toxicity from IDUR treatment of human herpes encephalitis and virus was isolated from brain tissue after a full course of treatment[39,40]. Similar results were obtained in rats and mice infected intracranially with herpes simplex or equine herpes virus[41,42,43]. The data suggest little reason for use of IDUR in such infections. A possible problem with IDUR is its previously reported potentiation of cytomegalovirus and adenovirus multiplication in tissue culture. Recent studies have added three unrelated RNA viruses, a DNA virus and a latent herpes virus to the list of viruses similarly potentiated in tissue culture by IDUR[44,45].

Ribavirin (Virazole - 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) - Broad-spectrum antiviral activity has been reported for ribavirin in tissue culture and in animal test systems. In mice, ribavirin was active orally against influenza A and B infections[46] and effective by IP or aerosol administration[47,48]. Other laboratory infections reported to be successfully treated were Sendai[49] and Friend leukemia in mice[50] and HA-1

in hamsters[49]. However, in two studies in man, ribavirin was inactive against challenge infections with influenza B[51] and influenza A[52]. In the latter study, amantadine HCl, used as a positive control, was effective in reducing disease.

Amantadine HCl (1-Adamantanamine HCl) - A complication of influenza A infections of man is a prolonged peripheral airway dysfunction. Amantadine HCl, which has anti-influenza A activity in tissue culture, animals and man, was tested in a double-blind study of natural influenza A (H_3N_2). A more rapid decrease in symptom score and reduction of severity of illness was reported for amantadine HCl treatment and, in addition, the drug-treated group had a significant increase in airway function within a week, whereas no such significant increase was found in the control group after 3 weeks[53]. There has been no progressive change in sensitivity of influenza A strains to amantadine HCl from influenza A/PR/8/34 (H_0N_1) to influenza A/Port Chalmers/1/73 (H_3N_2) as reported in a tissue culture study of methods to monitor influenza viruses[54], unlike the changes in sensitivity to antibody which occur with a change in influenza A type.

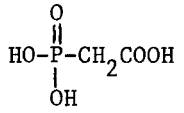
Rimantadine HCl (α-Methyl-1-adamantanemethylamine HCl) - Aerosol administration of rimantadine HCl to mice infected with influenza A (H_3N_2) virus provided a therapeutic effect, as measured by a decrease in mortality and an increase in mean day of death. The effect was measurable even when start of treatment was delayed up to 72 hours after infection, at which time histopathological lung lesions were already present[55]. Rimantadine HCl, spiroadamantane HCl and amantadine HCl were reported to show equal activity against influenza A infections of mice, but only the first two were shown to have activity on several rhinovirus strains in tissue culture[56].

Tromantadine HCl (Viru-Merz) - This N-dimethylaminoethoxyacetyl analog of amantadine has been reported to be effective by topical application against clinical herpes labialis, progenital herpes and generalized herpes infections of man with good to very good responses noted in 80% of patients[57].

Isoprinosine [4-(Acetylamino)benzoic Acid, 2-(Dimethylamino)-1-methyl Ester] - Most studies with prophylactic use of this agent have shown little activity. In one study, a therapeutic effect was reported for treatment started 48 hours after a challenge rhinovirus 21 infection in volunteers[58]. A combined study of prophylactic orange juice, simultaneous isoprinosine, and isoprinosine delayed for 42 hours after an intranasal challenge with rhinovirus 21 showed prophylactic/therapeutic activity on symptoms for all three treatments[59]. A possible mode of action for isoprinosine was suggested by a study which showed it caused an elevation in polyadenylic acid which was hypothesized to eventually stimulate the immune response[60].

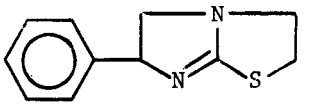
Phosphonoacetic Acid (PAA) - Additional trials with this interesting compound have confirmed its topical activity against herpetic keratitis in rabbit eyes[61,62] and against herpes infections in mice[63,64]. Repeated passage of a herpes virus in hairless mice treated with PAA has resulted in the isolation of a mutant resistant to PAA but not to ara-A[65]. In

addition to topical efficacy, PAA administered IP to mice and hamsters was effective against herpes encephalitis and IV administration to monkeys inhibited virus-induced neurological symptoms[66]. This agent appears to be safe and effective in laboratory studies and clinical trial results will be of interest.



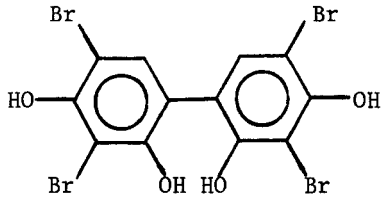
Disodium Salt

Levamisole HCl (Levo-tetramisole) - This anthelmintic agent has also shown activity against bacterial and viral diseases. Clinical trials with long-term treatment (up to 5 months) indicate that it was effective against persistent herpes simplex infections[67], aphthous stomatitis[68] and recurrent herpes labialis infection refractory to other treatment[69]. A suggested mode of action for the compound was enhancement of phagocytosis by macrophages[70].

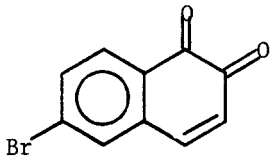


Vitamin C (Ascorbic Acid) - Disagreement on the role of ascorbic acid for the prevention and treatment of the common cold continues. One review of the experimental data concluded that high-level intake of ascorbic acid significantly decreased illness due to the common cold[71]. A second review of the data concluded that no reproducible pattern of efficacy could be shown for high-level ascorbic acid intake[72]. Several double-blind relatively well controlled studies in volunteers administered varying doses of vitamin C showed at best only minor effects on frequency and duration of colds, with no difference between high (3 grams per day) and low (200-400 mg per day) doses[73,74,75,76].

Tebrofen (Tebrophen) - A protective index of 51-60% against natural A/Hong Kong/68 and A/Port Chalmers/72 influenza infections was obtained by intranasal instillation of tebrofen ointment[77].



Bonaphthon - This compound was reported to provide significant protective and therapeutic activity against experimental pneumonia of mice and herpetic keratitis of rabbits[78].



Co-trimoxazole (Sulfamethoxazole plus Trimethropin) - Early treatment of children with this antibacterial agent was reported to cause herpes simplex

lesions to disappear and reduce recurrence of attack with the activity being reversed by penicillin[79].

Rifampin (Rifamycin) - Based on its reported activity in tissue culture against pox viruses and cytomegalovirus[80], this antibacterial agent was used for the treatment of subacute sclerosing panencephalitis in eight children but was completely ineffective[81].

ICN 4793 (3-Deazaguanosine) - Of a number of deazaguanosine derivatives tested, ICN 4793 was reported to provide the greatest protection to mice infected with influenza B virus or parainfluenza virus[82].

Among others with activity, CMA (10-carboxymethyl-9-acridanone) has *demonstrated* broad-spectrum antiviral activity by IP administration in mice[83], and 2,3-dihydroxy-6-bromopyrazino(2,3-b)pyrazine was shown to be active therapeutically against herpetic keratitis in rabbits as well as having broad-spectrum antiviral activity in tissue culture[84].

Prophylactic and therapeutic activity against experimental influenza A and B virus infections of mice was reported for several of a series of 84 quinolinehydrazones synthesized for structure activity studies[85] and for a number of benzamidine derivatives which possibly owed part of their efficacy to an anti-inflammatory effect[86].

Virus Inactivators - In laboratory test systems and in clinical challenge studies where the time of virus inoculum and drug treatment could be controlled, a number of virus inactivators have been effective. Among these, calcium elenolate, a potent inactivator, was found effective in preventing influenza A/PR/8/34 infections of hamsters when given intranasally within 10 minutes of virus challenge[87]. Another virus inactivator, silver sulfadiazine[88], prevented herpetic keratoconjunctivitis and encephalitis in rabbits when given within a few minutes of infection[89]. IP injection of the diuretic mersalyl (Salyrgan) was reported to reduce disease in mice infected with Coxsackie A and B viruses if given within an hour of infection[90].

Photodynamic Inactivation - The use of neutral red (NR) and light as a therapeutic treatment of herpes virus lesions has provided variable results. As reported in a recent communication, no beneficial effect was observed during a placebo-controlled double-blind clinical study of the effect of NR and light on 170 episodes of recurrent herpes infections[91]. Tissue culture studies suggested that herpes hominis was only inactivated by NR and light at concentrations of NR which were toxic to the cells, and the authors concluded that any observed effects from this procedure in treatment of disease may have been due to cell toxicity[92]. A different procedure, described as effective, was exposure of herpes virus lesions to UV irradiation (340-380 nm) after photosensitization with 8-methoxypsoralen[93].

Interferon and Interferon Inducers - Interferon research continues at an expanding rate. For this report, only a few of the recent highlights will be covered, in particular in reference to human clinical studies. The most recent advances in this field were covered exhaustively at the symposium "Antivirals with Clinical Potential" held August 26-29, 1975, at Stanford

University and the "International Symposium on the Clinical Use of Interferon" held October 1 and 2, 1975, at Zagreb, Jugoslavia, as well as being covered in recent reviews[3,94].

Many studies over the past years have demonstrated the efficacy of interferon and interferon inducers in the control of experimental virus diseases in animals, but the marked difference in responses with different animal hosts indicates that trials in man are necessary to determine the true value of these preparations.

Human leukocyte interferon (HLI) was effective in preventing herpes in monkeys and was tested in 84 patients for prevention of recurrence of herpetic keratitis[95]. At the time of the report, no ocular toxicity had been observed and 26 recurrences, 12 in the HLI and 14 in the placebo groups, had occurred. Another clinical trial of dendritic keratitis suggested that HLI treatment alone prolonged the disease course but that HLI plus cauterization was more effective than cauterization alone[96]. Better results were reported for the use of HLI for prevention and therapy of a number of virus-caused clinical diseases including labial and genital herpes[97]. To date the data on HLI is too preliminary to determine its potential efficacy, and many more well-controlled clinical studies are necessary.

The production of nasal interferon (NI) by topical application of N,N-dioctadecyl-N',N'-bis(2-hydroxyethyl)propanediamine (CP-20961) has been demonstrated[98]. Three different multiple-dosage schedules to volunteers infected with a rhinovirus challenge produced a consistent reduction in symptoms, but had no effect on the rate of infection. Treatment with CP 20961 of volunteers infected with influenza A/England/42/72 (H_3N_2) showed no difference from placebo-treated volunteers and induced NI titers were low[99]. A related, more potent interferon stimulator in animals, N,N-dihexadecyl-m-xylenediamine (CP 28888), was tested by nasal spray in thirty volunteers challenged with rhinovirus 13. Induced NI titers were lower than those reported for CP 20961 in contrast to the animal studies and respiratory illness occurred in 8/15 placebo subjects (2 febrile) and 9/15 treated subjects (1 febrile)[100]. This further indicates that in this area animal results do not correlate well with those obtained in human studies.

Tilorone HCl is an oral interferon inducer which has been effective against viral infections in laboratory animals. A single oral dose of 750 mg to 4 volunteers inoculated intranasally 24 hours later with rubella virus did not prevent clinical infection but shortened the illness and suppressed virus shedding and antibody response compared with placebo controls[101].

Topical application of polyinosinic-polycytidylic acid was found to be without effect for the treatment of localized herpes zoster infections of children with cancer[102]. It is possible that this mode of application did not stimulate interferon production sufficiently to generate clinically useful concentrations of interferon.

Summary - Antiviral chemotherapy has left the laboratory and is now established in the clinic with useful agents available. It has been relatively easy to demonstrate antiviral activity in model laboratory systems with every aspect controlled, and more difficult to translate this *into* clinically effective treatments. The model systems are useful as guides, but clinical trials are required. Unfortunately many of the clinical trials have not been well controlled and enthusiasm for a new antiviral agent has died out when controlled double-blind trials were carried out. With the many agents now being run in clinical trials, it would be of great value to run controlled studies from the start in order to ascertain their true therapeutic value as quickly as possible.

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Section IV - Metabolic Diseases and Endocrine Function

Editor: Hans-Jürgen Hess, Pfizer Inc., Groton, Connecticut

Chapter 15. Immunosuppressive and Immunostimulatory Agents in Rheumatoid Arthritis

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Introduction - During the past few years, the use of immunosuppressive therapy in rheumatoid arthritis has become widespread and reports on beneficial effects of immunostimulatory agents such as levamisole, BCG (Bacillus Calmette-Guerin), and transfer factor in rheumatoid arthritis have begun to appear in the literature. In this chapter, the theoretical basis of immunosuppression and of immunopotentiation for the treatment of rheumatoid arthritis is reviewed, followed by brief summaries of recent literature on the biological and clinical effects of individual agents. Comprehensive reviews on immunosuppressive therapy in rheumatoid arthritis,[1,2] the role of cellular immunity in rheumatoid arthritis,[3] and evidence for an infectious etiology of rheumatoid arthritis[4] have appeared. The proceedings of a symposium on mechanisms of tissue injury in rheumatoid arthritis have been published.[5]

Immunosuppressive Therapy

Concepts and Rationales - Immunosuppressive therapy for rheumatoid arthritis is based on the generally accepted view that immunologic events underlie the pathogenesis of this disease. There is ample evidence indicating that immunological phenomena are commonplace in tissues of patients with rheumatoid arthritis. A high level of activity of B-lymphocytes is indicated by (a) the demonstration of brisk synthesis of IgG, and to a lesser extent IgM and IgA, by rheumatoid synovial tissue;[6,7] (b) the deposition in the synovial membrane of complexes of IgG, IgM, IgD and complement,[8,9] and (c) the depressions of synovial fluid levels of complement components C_3, C_4, properdin and properdin factor B, indicating activation of both classical and properdin pathways[10] presumably by immune complexes. There is no longer any doubt that the rheumatoid synovium acts as a lymphoid-like organ producing large quantities of immunoglobulins, and that immune complexes consisting of aggregated IgG and rheumatoid factor of the IgM or IgG class are deposited in both the synovium and the synovial fluid. The role that these complexes play in the pathogenesis of rheumatoid arthritis is less clear. The complexes can produce tissue damage via interaction with complement and phagocytic cells. Significant numbers of polymorphonuclear leukocytes are present in rheumatoid joint fluid but not in the deep layer of the synovium or in the pannus. The synovial fluid polymorphonuclear leukocytes, which have been shown to contain immune complexes, contribute to the high levels of lysosomal enzymes found in the rheumatoid synovial fluid; these include β-glucuronidase, acid phosphatase, β-galactosidase, and cathepsin. The ex-

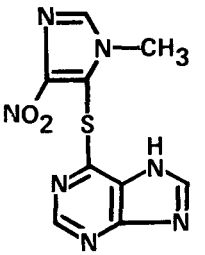
tent to which these enzymes participate in the degradation of joint components has not been clearly established. Mononuclear phagocytes are present in the sublining layers of the synovium and in the pannus which is a granulation tissue composed of fibroblasts and mononuclear cells. These cells have recently been shown to be capable of ingesting immune complexes[11] and to release lysosomal enzymes in vitro.[12]

Evidence is accumulating that cell-mediated immune mechanisms may play a role in the pathogenesis of rheumatoid arthritis. Substances displaying biological and chromatographic properties similar to lymphotoxin, migration inhibition factor (MIF), and blastogenic factor are found in synovial effusions of patients with rheumatoid arthritis.[13,14] Peripheral blood lymphocytes from patients with rheumatoid arthritis, upon exposure to autologous IgG, have been shown to undergo blast transformation[15] and to release MIF and MEF (migration enhancement factor).[16] Prolonged thoracic duct lymphocyte drainage appears to lead to clinical improvement.[17] Therefore, events characteristic of cell-mediated immunity may be taking place in the rheumatoid joint. These events may play a role in the pathogenesis of chronic synovitis. Alternatively, they may be secondary to the disease process.

The beneficial effects of immunosuppressive drugs such as cyclophosphamide and azathioprine in rheumatoid arthritis have been considered by some to be indicative of the occurrence of abnormal immune responses in the disease. However, the mechanism(s) of antiarthritic action of these drugs has not been elucidated conclusively and they are known to have antiinflammatory effects.[18,19] There is a lack of correlation between therapeutic efficacy and immune status of patients receiving immunosuppressive drugs.[1] It is entirely possible that the beneficial effects of these drugs in rheumatoid arthritis are, at least in part, the result of antiinflammatory actions.

In summary, there is ample evidence that immunologic events, both humoral and cellular, take place in the rheumatoid joint. The rheumatoid synovium synthesizes large quantities of immunoglobulin. The weight of available evidence favors the view that this local activity leads to complex deposition, cellular infiltration, release of lysosomal enzymes, lymphokines, and other mediators in both the synovium and the synovial fluid with inflammatory sequelae. Nevertheless, the pathogenic role of immunologic phenomena in rheumatoid arthritis, upon which immunosuppressive therapy is based, has not yet been rigorously established.

Azathioprine (1) is rapidly converted to 6-mercaptopurine in vivo and has a spectrum of activity generally similar to that of 6-mercaptopurine.[20] The general impression that azathioprine is a better immunosuppressive agent that 6-mercaptopurine has been questioned.[21] Both compounds act as purine analogues, thereby inhibiting nucleic acid biosynthesis and exerting a cytostatic effect on cells at the S phase of the cell cycle. Azathioprine has been shown to suppress the primary humoral immune response,[22,23] although the effect is highly



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dependent on timing.[22] It has also been reported to suppress cell-mediated immune response[24] and animal models of autoimmunity.[25]

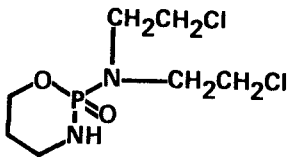
Azathioprine is the most widely used immunosuppressive drug in the therapy of rheumatoid arthritis. Reports of controlled clinical trials of azathioprine in rheumatoid arthritis are in agreement that the drug, when given in sufficient quantity (1.0 - 2.5 mg/kg per day) and for a sufficient period of time (3 months or longer), reduces the symptoms of the disease.[26-28,2] The degree of improvement is considered to be comparable to that achieved with conventional gold therapy.[28] Beneficial effects can be expected in more than 50 per cent of the patients with azathioprine a few months after starting treatment.[1] Long-term follow-up studies (40 months) have shown that the drug continues to exert a suppressive effect on the disease when administered over a prolonged period of time.[26] The drug, however, does not induce a lasting remission or slow the development of new erosions.[26]

The mechanism of action of azathioprine in rheumatoid arthritis is not understood. Immunosuppressive effects of azathioprine in man have been claimed and denied.[19,29-33] In studies of patients with rheumatoid arthritis, the drug has been reported to suppress immunoglobulin synthesis, but has no effect on the titer of rheumatoid factor, the responsiveness of lymphocytes to mitogens, or on the relative proportions of T-cell and B-cells.[2,29,34] The level of peripheral circulating lymphocytes in patients treated with azathioprine has been reported to be reduced[34] or normal.[35] The drug is known to have antiinflammatory activity[18,19] which may be related to its effect on the level of circulating polymorphonuclear leukocytes and monocytes.[19,36] Although the beneficial effects in rheumatoid arthritis may be due to the antiinflammatory activity of azathioprine, clinically the drug has a more pronounced therapeutic effect than the currently available non-steroidal antiinflammatory agents.

Azathioprine, at dosages used for the treatment of rheumatoid arthritis, does not induce severe bone marrow depression,[1,26] although leukopenia has been reported.[26] Other side effects such as nausea, alopecia and skin rashes occur with low incidence.[2,26,27] Unmasking of neoplasia is a well recognized risk of immunosuppressive therapy.[37] Additionally, azathioprine usage is associated with an increased incidence of chromosomal abnormalities in patients on long-term therapy.[26] It is mutagenic,[38] and leads to an increased incidence of tumors in mice.[39] Unlike cyclophosphamide, azathioprine does not cause azoospermia.[28]

Cyclophosphamide (2), an alkylating agent, has been shown to inhibit both humoral [23,40] and cellular immunity[18,41] and to suppress the passive transfer of cellular immunity.[42] The drug must first be metabolically activated *in vivo*.[43-46] In mice, chickens, and man there appears to be a greater suppressive effect on B-cells than T-cells.[47-49]

The exact mechanism(s) underlying the immunosuppressive action of cyclophosphamide is unknown. As an alkylating agent, the drug interferes with cell metabolism and exerts a cytotoxic effect independent of phases of



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the cell cycle. It appears not only to interfere with early events in the generation of the immune response but also to have a direct effect on B and T effector cells.[50]

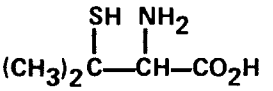
Cyclophosphamide has been shown in controlled clinical trials to be effective in the therapy of rheumatoid arthritis at "high" doses (1.5 to 3.0 mg/kg per day),[51,52] but not at "low" doses (0.7 to 1.4 mg/kg per day).[53-55] The toxic side effects of the drug were almost as great at the "low" dose.[53] In a more recent study, Hurd and Ziff[56] reported "significant improvement" in patients with rheumatoid arthritis receiving 1.0 to 1.5 mg/kg per day. Among various studies the incidence of improvement of rheumatoid arthritis patients treated with cyclophosphamide ranged from 75% to 100%.[51,52,57] Patients on cyclophosphamide therapy appeared to show less radiological joint deterioration than patients treated with gold.[28]

The mechanism of action of cyclophosphamide in rheumatoid arthritis is not understood. The drug appears to exert suppressive effects on some parameters of the immune response which may be of primary importance. There is a lack, however, of correlation between therapeutic efficacy and immune status by any of the parameters measured to date. Studies of cyclophosphamide-treated patients with rheumatoid arthritis demonstrated decreased numbers of circulating lymphocytes.[56,58] Whether this represents a true indication of immunosuppression is questionable. Cyclophosphamide-treated patients showed normal responses upon skin testing with common environmental antigens, but their lymphocyte response to stimulation by phytohemagglutinin and pokeweed mitogen was reduced.[59] Most investigators reported a decrease in both serum immunoglobulin levels, particularly IgG,[51,56,60] and in rheumatoid factor titers.[51,60]

Notable adverse side effects of cyclophosphamide include alopecia, hemorrhagic cystitis, azoospermia, and sustained lymphopenia. Alopecia occurs in about 50% of patients at the therapeutic dose.[51,52] Hair growth frequently returns even while the drug is continued at the same dose level and total alopecia is rare.[1] Hemorrhagic cystitis occurs at about the same frequency as alopecia.[51,52,57] Currey *et al*[28] recently reported that all male patients receiving cyclophosphamide developed azoospermia. The potential long-term adverse effects, particularly with regard to development of neoplasia and facilitation of infection, may very well override the usefulness of cyclophosphamide in rheumatoid arthritis. Cyclophosphamide is a known mutagen.[61] It has been clearly shown that long term cyclophosphamide therapy leads to tumors in mice.[39] Pollock *et al*[63] reported two malignancies in rheumatoid arthritis patients taking cyclophosphamide. Recently, a recurrence of a previously treated melanoma was reported in a patient on cyclophosphamide.[64]

D-penicillamine (3), an orally active chelating agent has been used in the

treatment of heavy-metal intoxications, cystinuria and Wilson's disease.[64] The use of this drug in the treatment of rheumatoid arthritis has recently brought it into prominence.[62] There is a general impression that D-penicillamine modulates immune responsiveness despite lack of clear experimental evidence. Altman and Tobin[66] reported that rabbits treated with DL-penicillamine beginning one day prior to intravenous injection of human serum albumin showed suppression of antibody production. Administration of the drug for 28 days before sensitization resulted in an accelerated onset of immune response.[67] On the other hand, Hubner and Gengozian[68] reported that treatment of mice with DL-penicillamine for 3 weeks before the injection of *S. typhosa* antigen resulted in an overall reduction in antibody formation while treatment immediately after antigen injection for 8 consecutive days had no effect. Treatment of rats with D-penicillamine appears to have no effect on the development of an immune response to sheep red blood cells.[69] D-penicillamine enhances the secondary lesions of adjuvant arthritis when administered to animals with established disease,[70] whereas daily administration of the drug beginning 4 days prior to the injection of adjuvant has no effect on the development of this experimental disease.[69] In vitro, D-penicillamine has the ability to depolymerize and inactivate proteins through its sulfhydryl radical.[129]



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The results of a double-blind controlled study of D-penicillamine for the treatment of rheumatoid arthritis conducted by the Multicenter Trial Group[71] in the United Kingdom seemed to be in agreement with the findings of various uncontrolled studies[72-74] that the drug benefits patients with acute, severe rheumatoid arthritis. A comparative study showed that D-penicillamine and gold were equally effective.[74]

The mechanism of action of D-penicillamine in patients with rheumatoid arthritis is unknown. The early hypothesis of depolymerization of preformed rheumatoid factor[75] is no longer considered valid.[69,73,76] There is no clear evidence that D-penicillamine exerts its action in rheumatoid arthritis by suppressing an immune response. Falls in latex titer, levels of IgG, IgM, IgA and complement fraction C_3 were shown in patients treated with D-penicillamine, but these changes did not bear any relation to clinical improvement and are believed to be due to some non-specific effect such as inhibition of protein synthesis.[73,76,77] D-penicillamine therapy was reported to produce no significant change in anti-nuclear factor,[77] and no suppression of responsiveness to keyhole limpet haemocyanin or tuberculin.[77] A reduction in peripheral blood lymphocyte count was reported in patients treated with D-penicillamine.[78] It is questionable whether this represents a true indication of immunosuppression. Jaffe *et al*[79] reported that D-penicillamine, at concentrations producing no effect on host cell RNA or protein synthesis produced a marked inhibition of virus specific RNA and protein synthesis and speculated that this may be relevant to its therapeutic effect. Untoward effects include alterations in taste, stomatitis, gastrointestinal distress, unpleasant skin odor, and more serious, agranulocytosis, thrombo-

cytopenia, and nephritis. Bacon *et al*[80] reported recently that immunofluorescent and electron-microscopy studies of renal biopsies from 14 rheumatoid patients on D-penicillamine showed an immune complex type of nephropathy without exception. There also is a report of the development of myasthenia gravis in four patients with rheumatoid arthritis after taking penicillamine.[81]

Immunopotentiation Therapy

Concept and Rationales - The immunopotentiation approach is based on the belief that persistent infection (virus, mycoplasma or bacteria) is involved in the pathogenesis of rheumatoid arthritis and/or that the disease is, on the whole, a manifestation of autoimmunity brought about by a deficiency in suppressor T-cell function. However, all attempts made to isolate infectious agents from rheumatoid synovium or fluid have been met so far with failure. In recent years, increasing attention has turned to a viral etiology of rheumatoid arthritis despite lack of confirmatory evidence.[4,82] There is a growing appreciation of "atypical" forms of viral-host cell interaction that may be relevant to rheumatoid arthritis whereby the virus maintains a persistent association with the cell and may transform cells or cause chronic degenerative diseases rather than those of the acute and self-limiting types. In the New Zealand mouse disease, for example, it appears that a vertically transmitted type-C virus initially replicates in the thymus and causes defective cellular immunity.[83] The defective cellular immunity allows continued viral replication and antigen expression which induces an increasing humoral response directed not only at viral and altered host antigens, but also, because of the lack of suppressor T-cells, at other unrelated host and foreign antigens. Immunologic disease then occurs as a result of immune complex deposition.[84] A similar sequence of events could be involved in rheumatoid arthritis. For a more detailed discussion of an infectious etiology of rheumatoid arthritis, the reader is referred to excellent recent reviews by Hamerman.[4,5]

Rheumatoid arthritis can be considered as an autoimmune disease in as much as the IgM immunoglobulin of rheumatoid factor is an antibody against autologous, uncoiled IgG. Hypotheses concerning an apparent predisposing role of T-cell deficiency for the appearance of autoimmunity in experimental animals and in man have been reviewed by Allison.[85] Autoantibody formation may result from a deficient or an impaired T-cell function, thereby allowing unregulated B-cell populations to function unchecked and to produce antibodies against self-constituents. There are several lines of evidence that suggest a deficiency or an impairment of cellular immunity in rheumatoid arthritis. A significant peripheral lymphopenia occurs in patients with rheumatoid arthritis.[86] Most investigators agree, however, that the proportions of T-cells in the peripheral blood of patients with rheumatoid arthritis is similar to that in normal individuals.[3,86-88] The proportion of B-cells in peripheral blood of rheumatoid arthritis patients has been reported to be high or normal.[3,87,88] Particularly patients with rheumatoid arthritis of long duration, seem to have an impaired delayed hypersensitivity response as measured by skin testing with antigens such as PPD, mumps, *Candida albicans*, or DNCB.[89-91] The responsiveness of peripheral

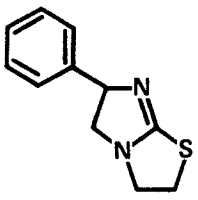
blood lymphocytes of patients with rheumatoid arthritis to stimulation by phytohemagglutinin, concanavalin A, and pokeweed mitogen was reported to be depressed,[92] while the response of synovial fluid cells to stimulation by phytohemagglutinin and concanavalin A were reported to be indicative of the presence of T-cells.[93] Interpretation of these results is made difficult by the fact that little is known about the response of synovial fluid cells in other inflammatory, non-rheumatoid joint diseases. It is possible that all these apparent manifestations of an impaired immune response may be reflections of a chronic disease state or long-term drug therapy, rather than indicators of a fundamental immune abnormality.

BCG - General nonspecific immune potentiation can be achieved by the administration of Bacillus Calmette-Guerin (BCG) which affects both cell-mediated[94] and humoral immune responses.[95,96] It has been shown to cause not only an increase in the number of functioning lymphocytes and macrophages,[96,97] but also increased activity of individual cells.[98] The agent was reported recently to delay the onset and reduce the frequency and severity of adjuvant induced arthritis in the rat when administered before the injection of adjuvant.[99]

A preliminary report indicating improvement of rheumatoid arthritis in patients given BCG[100] has received anecdotal support.[101] BCG has been shown to increase immune complex clearance by macrophages,[98] enhance nonspecific lymphocyte-mediated cytotoxicity and macrophage-mediated cytolysis,[102] and increase the production and release of interferon *in vivo*. One or more of these mechanisms may be pertinent to the reported beneficial effect in patients with rheumatoid arthritis.

BCG vaccination has been performed in more than 500,000,000 people[103] and serious complications are rare.[104] However, BCG has multiple actions on the immune response, some of which, the increased antibody production for example, may aggravate rheumatoid arthritis and the overall effect on the disease cannot be easily predicted.

Levamisole (4), an anthelminthic, has been used extensively in humans and animals for nematodal infestations with few side effects.[105] Recently, the drug has been reported to stimulate some aspects of both cellular and humoral immunity in experimental animals[106-109] and to restore cutaneous delayed hypersensitivity reactions in anergic elderly humans[110] and in some patients with cancer.[111-113] In a recent report, Huskisson *et al*[128] stated (no data given) that the drug had no effect in carrageenan pleurisy or on the primary lesions of adjuvant-induced arthritis. But, the secondary lesions of adjuvant arthritis were more severe in animals receiving levamisole, presumably due to an enhanced cell-mediated immune response (no data given). The mechanism by which levamisole enhances the immune response is not known, although mediation by cyclic nucleotides has been



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suggested.[114] The drug has been reported recently to cause the production or release in mice of a heat-labile, non-dialysable humoral factor. The factor stimulates the reticuloendothial system as manifested by increased carbon clearance presumably via activation of existing phagocytic cells.[115]

Several preliminary reports of studies of levamisole in patients with rheumatoid arthritis have appeared,[116-119,128] and, with one exception,[118] all reported beneficial effects. Untoward side effects included stomach upset, transient diarrhea, alteration in the sense of smell, irritability, insomnia, and reduced white blood cell count.[120,128] A serious adverse reaction, severe leukopenia, in patients on long-term levamisole therapy has been reported recently.[121]

Although it is generally thought that the favorable outcome of levamisole therapy may be due to stimulation of the cell-mediated immune response, there is as yet no clear supportive evidence. The clinical course of patients with rheumatoid arthritis treated with levamisole has been reported to correlate with responsiveness of lymphocytes from these patients to antigenic stimulation.[117] A correlation between pain relief and delayed skin test sensitivity to common antigens including PPD has been claimed,[70] while another group found a correlation between clinical improvement and lymphocyte stimulation.[117] Levamisole has been shown to depress hyaluronic acid production in vitro by cultured synovial cells. It was suggested that this action may contribute to the antiarthritic effect.[122]

Transfer factor is a material obtained from filtered enzyme-treated extract of human leukocytes and contains a number of components including nucleotide and peptide units. Aside from its dialyzability and apparent resistance to degradation with deoxyriboneuclease, pancreatic ribonuclease and trypsin,[123] little is known about the chemical nature of transfer factor. Although there is agreement that transfer factor endows skin test-negative recipients with the ability to develop the manifestations of some of the delayed hypersensitivity responses of the donors, there is little direct information on the mechanism of this phenomenon or on the nature of the active component(s). The properties and activities of transfer factor have recently been reviewed.[124,125] Kass *et al*[126] studied the effect of transfer factor in three patients with severe juvenile rheumatoid arthritis and reported a beneficial effect and the conversion to positive of a previously negative delayed hypersensitivity response. On the other hand, Maini *et al*[127] reported that the beneficial effects of transfer factor were entirely comparable to placebo.

Conclusion - It has become abundantly clear that both humoral and the cellular immunologic events are commonplace in joints of patients with rheumatoid arthritis. Although the weight of evidence favors the view that immunologic mechanisms play a pathogenic role, the possibility remains that these immunologic manifestations may represent secondary phenomena in an ongoing disease process. There is no longer any doubt that cyclophosphamide and azathioprine exert a suppressive effect on rheumatoid arthritis. The relevance of immunosuppression to the favorable outcome of patients treated with these drugs has been implied, but clear supportive evidence is

lacking, and it is not unlikely that the beneficial effect of these drugs may be due, at least in part, to their antiinflammatory activity. It has become clear that immunosuppressive therapy, at least with the currently available drugs, is not curative as was initially hoped. The risk/benefit ratio of long term therapy appears high considering the adverse effects, particularly the development of neoplasia and facilitation of infection. Although immunopotentiation represents an alternative, we know little about its potential adverse consequences. The discovery of specific and non-toxic immune modulatory agents is a worthy goal. The task is made difficult by our incomplete knowledge of the immune system and the lack of clear understanding of the specific immunologic defect which underlies rheumatoid arthritis. In the excitement generated by favorable reports of levamisole therapy, it may be prudent to remember that the theoretical basis for either immunosuppression or immunopotentiation has not been rigorously established and other approaches such as those based on functions of inflammatory cells, mediators of inflammation, and complement should not be neglected.

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Chapter 16. Steroids

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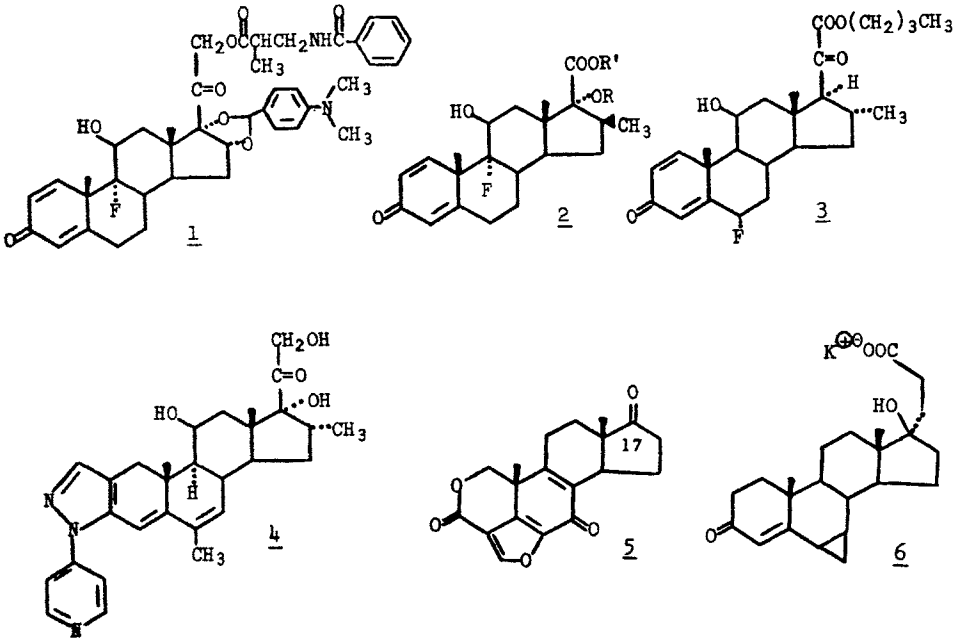
Introduction - In this chapter the recorded literature since 1972, the last review in ANNUAL REPORTS, is reviewed with particular emphasis on papers published during 1975. The Proceedings of the Fourth International Congress on Hormonal Steroids were published in 1975 and provide excellent background material for many of the areas discussed.[1] The chemistry of steroids is reviewed on a yearly basis[2], and the first of a biennual series of critical reviews of steroid research has appeared.[3]

Corticosteroids - A review on antiinflammatory steroids covering developments during the period 1964-1972 was published.[4] Much of the literature of succeeding years concerned clinical trials of previously reported structures. Three of the most potent fluorinated corticosteroids described were betamethasone dipropionate (Diprosone®)[5], halcinonide (Halog®)[6], and clobetasol propionate (Dermovate®).[7] Hydrocortisone 17-butyrate compared favorably to betamethasone valerate and fluocinolone acetonide[8], suggesting that fluorination is not necessary for high topical activity. The use of the potent topical steroids betamethasone valerate[9] and beclomethasone dipropionate[10,11] as aerosol inhalents may represent a significant medical advance in the treatment of asthma and allergic rhinitis. Finally, Δ^6-6-chloroprednisolone (cloprednol) exerted twice the antiinflammatory potency of prednisolone in rheumatoid arthritis, had a short plasma half-life, and slightly less adrenal suppression than prednisolone[12], suggesting clinical utility as once-a-day or alternate-day therapy.

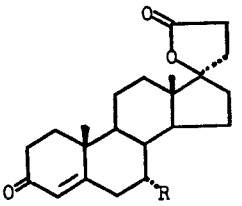
In other developments, a marked dissociation of systemic from local effects was ascribed to 1.[13] In the rabbit 1 showed ocular antiinflammatory activity greater than betamethasone valerate, and lower intraocular hypertension, weight loss, and mortality following high doses.[13] High topical activity in the McKenzie vasoconstriction assay was described for a series of diesters of 17α-hydroxyandrostane 17β-carboxylic acids[14] (2). The most interesting compounds were the 17α-propionyloxy, 17β-methoxy- and 17β-halomethoxy-carbonyl derivatives.[14] A steroid containing a 21-carboxylic acid, fluocortin butyl (3), reportedly showed significant topical vasoconstriction on human skin, with little or no systemic activity in rats.[15] High local and low systemic activity was also reported for a series of 14α,17α-alkylidenedioxycorticosterone 21-esters.[16] A similar separation of systemic from local effects, as well as clinical efficacy against psoriasis, was reported for 4.[17] The influence of the 6-azido-6-ene group on antiinflammatory potency was studied in the rat.[18] Systemic potency of 9α-unsubstituted corticosteroids was increased 5-8 times by this modification whereas potency of 9α-fluorocorticosteroids was largely unaffected.[18] In standard rat antiinflammatory assays, oral 5 (11-desacetoxywortmannin, a metabolite isolated from culture filtrates of Penicillium funiculosum Thom) was more potent than non-steroidal agents tested, although less potent than dexamethasone.[19] Introduction of a



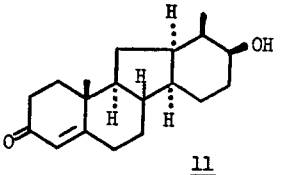
corticosteroid side chain at C_{17} abolished antiphlogistic activity. The antiinflammatory activity was not a consequence of adrenal gland stimulation.

Aldosterone antagonists - Potassium prorenoate (6) is more potent orally than spironolactone (7) in both dogs (4.6x) and rats (3x) but has otherwise a similar pharmacological profile.[20] In man 6 was significantly more potent than 7 in elevating Na/K ratios and in conserving potassium.[21] The 1,2-dehydro analogue of 6 was also a potent, orally active aldosterone antagonist in rats without any anti-androgenic effects.[22] Also reported[23] were a series of 7α-carboalkoxy derivatives with the ethyl (8) and isopropyl (9) esters being the most potent in the DCA treated, adrenalectomized rat. The rac-19-nor-18-methyl isomer of 10 is reportedly[24] more active than 7 in diuretic action while the 16β-OH derivative of 10 has only 1/7th the anti-aldosterone potency of 7 in rats.[25] An attempt[26] to confer oral activity to etiojervane 11 by adding a 7α-SAc group was unsuccessful. 15-Ketoprogesterone and its 1,2-dehydro analog are potent aldosterone antagonists comparable in potency to spironolactone; however introduction of a 6β,7β-methylene group lowered subcutaneous potency and abolished oral activity.[27] Removal of the 11-oxo function[28] from glycyrrhetic acid changed the weak mineralolocorticoid action of that compound to potent anti-DCA activity in the rat.

A new in vitro assay for aldosterone antagonists has been developed[29]



7, R=SAc
8, R=COOEt
9, R=COOi-Pr
10, R=H



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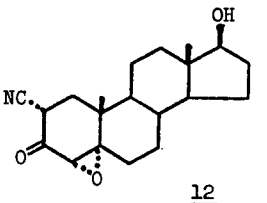

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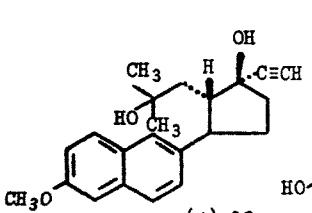
using aldosterone receptors (type I) from rat kidney cytoplasm. Of 24 known aldosterone antagonists 9 had the highest affinity for this receptor (70% that of aldosterone) with (11) second (35%).[29]

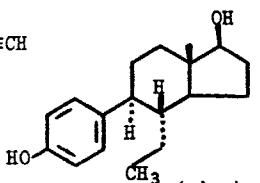
Three 16-oxygenated steroids have been implicated in low-renin hypertension in man.[30,31] 16α-Hydroxy-dehydroepiandrosterone and the 16-keto, 17β-hydroxy isomer, found in large amounts in urine, had 1/40 the mineralocorticoid potency of aldosterone in rats[31], while 16α,18-dihydroxy deoxycorticosterone, excessively secreted by adrenal tissue, enhanced the action of aldosterone.[30] Compound 12 which inhibits the conversion of pregnenolone to progesterone (and hence aldosterone) has been successfully used to control primary hyperaldosteronism.[32]

Estrogens and anti-estrogens - A review on anti-estrogens was published.[33] The modes of action of estrogens, estrogen receptors, and new assay techniques were described in the Abstracts[34] and Proceedings of the Fourth International Congress on Hormonal Steroids.[1]

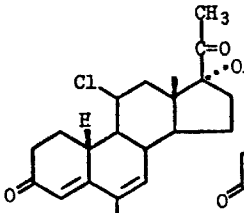
In rats, dimeric estradiol-ethynodiol and dimeric ethynodiol showed prolonged estrogenic and anti-estrogenic activity, respectively.[35] The bifunctional alkylating steroid 2,4-bis (bromomethyl) estrone methyl ether lacked estrogenic activity but the anti-estrogenic effect was greater and of longer duration than that of estrone methyl ether.[36] Of a series of 70 labile 17-ethers of estradiol, the enol ethers of 3-pentanone and 5-9 membered cyclanones showed high oral uterotrophic activity in mice.[37] Cycloalkenyl ethers derived from 8-15 membered cyclanones showed high and prolonged parenteral uterotrophic activity.[38] The effect of scission of the 9,11 steroid bond on biological activity was investigated[38,39], where 13 was found to have antiestrogenic, antifertility, and antiinflammatory activity, with estrogenic activity approximately 0.003% that of estrone.[38] Orally administered 11-hydroxy-9,11-secoestradiol and the 3-methyl ether completely prevented implantation in rats.[39] A series of 5,6-secoestradiols was synthesized with 14 having the highest estrogenic and anti-implantation activity.[40] In addition, the corresponding 8β,9β-cis steroid showed significant antiimplantation and estrogenic activity, leading to the conclusion that the disposition of the aromatic ring with respect to rings C/D is not critical for receptor binding.[40] 11α-Methoxy ethynyl-estradiol (RU-16,117) was described as a potent anti-estrogen in the rat.[41] Its antiestrogenic activity was attributed in part to an inhibitory effect at the level of the pituitary.[41] The synthesis and biology of 17α-furylestradiol and dihydroequilin derivatives[42], and their lactone and anhydride conversion products[43], were published. The 17α-(3-furyl) analogs were 4-19 times as potent orally as mestranol in rats but less po-



(±) 13



(±) 14



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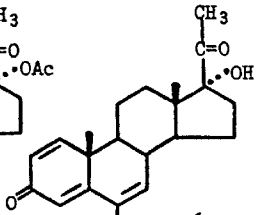

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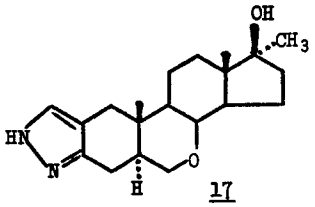
tent in mice.[42] Similarly a series of 7α,8α-epoxy-, methylene, and difluoromethylene estradiols were highly active orally in rats, but only weakly active in mice.[44] The use of receptor binding assays has been proposed for screening of agents with anti-hormone activity.[45]

Progestagens - The flow of publications in this area has declined considerably since the last review. Structure-activity relationships in a comprehensive series of analogs suggested removal of the 10-methyl group, introduction of an 11β-chloro group in 19-nor steroids, and introduction of a 16-methylene moiety are important for progestational activity.[46] However, although compound 15 demonstrated extremely high (162-226x progesterone) activity in rabbits, the contraceptive potential was not confirmed in preliminary clinical testing.[47] In studies correlating in vivo progestational activity with in vitro receptor binding, a series of 17α-ethyl-substituted pregnanes were found inactive in affecting implantation although they exhibited strong receptor binding in vitro.[48] Compound 16 which lacks progestational activity, exhibited moderate antiinflammatory activity, including activity in models mediated via delayed hypersensitivity. In contrast to cortisol which inhibits both T-S and 19-S antibody formation, 16 did not diminish the number of antibody-producing cells.[49]

Androgens - A general review of androgens, anabolic agents, and androgen antagonists was published.[50] 19-Nortestosterone homofarnesate (nandrolone homofarnesate; DA-1979) was 2-4 times more potent than nandrolone β-phenylpropionate as an anabolic agent, with lower androgenic effects, and longer duration of action.[51] Low oral antigonadotrophic, androgenic and anabolic activities were reported for a series of 7-oxaandrostane derivatives however, 7-oxa-winstrol (17) exhibited significant antigonadotrophic effect without anabolic or androgenic activity.[52] In a series of 19-nortestosterone derivatives, 7α-methyl-14-dehydro-19-nortestosterone was 100 to 1000 times as potent as testosterone in various tests.[53] Synthesis of some des-ring A steroids was described, with 18 showing a separation of androgenic and anabolic activities.[54] A class of 19-norsteroids having a 17α-ethinyl group but lacking the 17β-hydroxyl were described[55] which retained the progestational activity of norethindrone but lost its androgenic activity. Of a series of steroidal imidazole-1-carboxylic acid esters, the imidazole-1-carboxylate of 19-norethisterone showed increased progestational and decreased androgenic activity relative to the parent compound.[56] The synthesis of sulfur, selenium, and tellurium containing 5α-androstane derivatives was described, with 19 having 1/5 the



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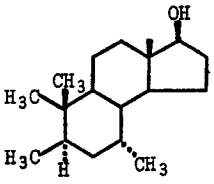

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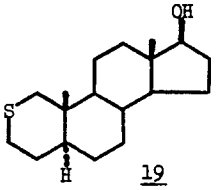

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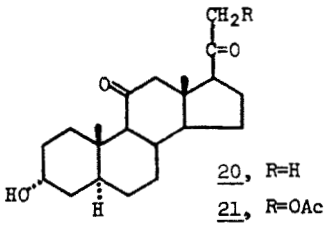
androgenic activity and nearly the same anabolic activity as testosterone.[57] The most potent compound among 16 testosterone 5α-reductase inhibitors tested in vitro was 4-androsten-3-one 17β-carboxylic acid.[58] The influence of structural changes and the ratio of androgenic and anabolic activities was examined in a series of 19-nortestosterone-related $\Delta^{4,9,11}$-triene-3-keto steroids, of which the hexahydrobenzyl ether had an anabolic/androgenic ratio of 10.[59]

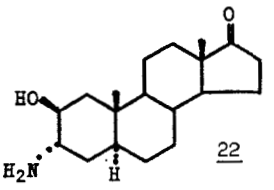
Steroid anesthetics and neuromuscular blocking agents - The steroid anesthetic Althesin® [20 + 21 (3:1) in Cremophor EL] has been successfuly introduced into clinical practice in England.[60] The vehicle has been connected with a few anaphalactoid reactions and therefore the search for a water soluble and/or more potent steroid has continued. Ring-A substitution seems critical for potency[61,62] since the 2β-Cl,2β-Me,2β-OEt and 2β-O-i-Pr derivatives of 20 were all considerably more potent than 20 in the mouse assay. The 21-CN derivative, although somewhat less potent than 21, forms a relatively stable water soluble Na salt.[62]

The amino steroid 22, a potent local anesthetic, had antiarrhythmic activity greater than lidocaine.[63] Further experiments[64] suggest a possible clinical use of this compound in the treatment of early arrhythmias following myocardial infarction.

The development and pharmacology of steroidal neuromuscular blocking agents was reviewed[65] and the chemical work related to the clinically useful muscle relaxant, pancuronium bromide (23), was reported.[66] Further work[67] to uncover an agent free of side effects and with a rapid onset of action has produced 24, which had only 1/5 the potency of 23 in grip strength studies in man but had a more rapid onset of action. The 17α-aza-D-homo-steroid, 25, was reported to possess similar properties.[68] An analysis of the 3-dimensional structures of steroidal and non-steroidal neuromuscular blocking agents[69] by X-ray diffraction analysis has resulted in some "structural rules" for potent neuromuscular blocking activity. These include a rigid molecular structure and two quaternary nitrogen atoms 10.8±0.3 Å apart. Batrachotoxin and some derivatives have been tested on rat phrenic nerve-diaphragm muscle preparations for depolarizing activity.[70] For maximum potency the 3α,9α-hemiketal link must be intact and a methyl-substituted 20α-pyrrole 3-carboxylate group is necessary.



20, R=H
21, R=OAc



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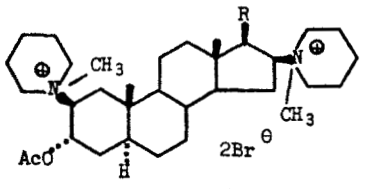
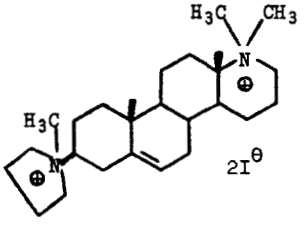


23, R=OAc
24, R=H



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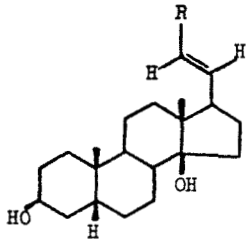
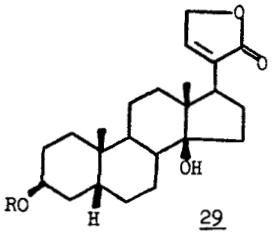
Cardenolides - The chemistry, pharmacology and structure-activity relationships in this area were extensively reviewed.[71] The butenolide lactone ring, previously thought to be essential for cardiotonic activity, has been replaced by certain α,β-unsaturated groups, e.g., 26 and 28, with little loss in potency.[72] However, activity is very sensitive to further structural changes, as for example even the ethyl ester 27 is much less potent than 26. Further work on the synthesis[73] and pharmacology[74,75] of isocardenolides of type 29 have been reported. AY22, 241 (29, R=glucose) has a more rapid onset of action, a greatly improved therapeutic ratio and its toxic effects are more rapidly reversible in animals than those of conventional cardenolides.[74] Structure-activity relationships concerning the steroid nucleus have also been reported. Modifications at C_{14} and C_{15}[76] and in ring-A[77] led to a uniform loss in potency while introduction of a 6α-methyl group into digitoxigenin 3β-acetate did not change cardiotonic activity.[78]



26, R=COOMe
27, R=COOEt
28, R=CN



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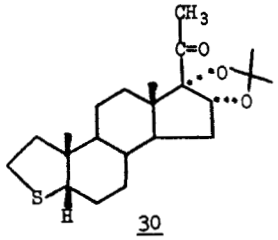

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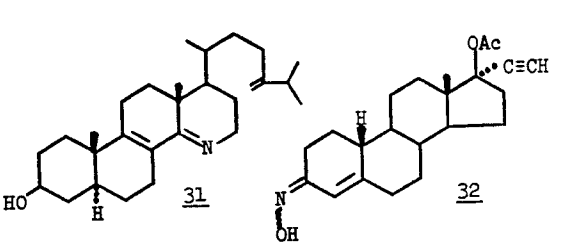

Heterosteroids - The unusual A-nor-3-thia steroid, 30 was reported[79] to exhibit vasoconstrictor activity in humans equivalent to betamethasone valerate. An interesting group of azasteroids with high anti-fungal activity have been isolated[80] from the crude fermentation broth of the fungi Geotrichum flavo-brunneum NRRL-3862. The major component of the complex, A25822B (31) was also the most active with MIC values in the range 0.0312-5.0 mcg/ml against Candida albicans and other pathogenic fungi. A series of oximino and 3-aza-A-homo androstenes were synthesized from norethindrone acetate, with the 3-oxime, 32, showing a 137-fold increase in anti-fertility potency in rats.[81] The 3-methoxime of norethindrone showed similar anti-fertility activity.[82]

Steroids and cancer - Only 20-40% of metastatic human breast cancers respond to hormone therapy. This rate can now be raised to 55-60% by selecting only those tumors which contain estrogen receptors.[83] Very recently it has been suggested that this rate can be further increased if the tumor also contains progesterone receptors[84] and preliminary clinical data support this view.[85]

One of the most effective steroids for the treatment of advanced breast cancer is 7β,17α-dimethyl-testosterone (calusterone)[86,87] which curiously had shown no activity in a number of animal cancer screens. It is an orally active, weakly androgenic steroid which, at 200 mg/day, was more effective than testolactone (1000 mg/day).[86]

The 21-chlorambucil ester of prednisolone (LEO 1031)[88] has twice the therapeutic index of an equivalent mixture of prednisolone and chlorambucil against the solid Wa256 carcinoma and is also reported active against human lymphocytic lymphomas. An interesting new type of "designed" anti-tumor agent is 33 which contains the α-methylene-γ-lactone group present in many anti-tumor compounds of plant origin.[89]

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Chapter 17. Peptide Hormones

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Introduction — Peptide hormones reviewed in this year's chapter are of other than hypothalamic or pituitary origin[1]. The most interesting developments include: (a) the confirmation and extension of the general concept of hormone biosynthesis by enzymatic cleavage of prohormones and biologically inactive pre-prohormones; (b) the isolation of endogenous opioid agonists; (c) sequence analysis of thymopoietin, a T-cell stimulating thymic factor; (d) the first truly total synthesis of insulin with sequential formation of the three disulfide bonds, and (f) a partial separation of insulin and glucagon suppressing effects in a somatostatin analog. Readers are referred to books[2-4] and reviews[5,6]. A new nomenclature for peptide hormones was recommended by the IUPAC-IUB Commission[7,*].

Endogenous Opioid Agonists — Several years of search for a putative factor in brain, which activates opiate receptors, has culminated in the isolation of two pentapeptides, Met-enkephalin [Ia] and Leu-enkephalin [Ib] from guinea pig brain[8]. Both have been synthesized and showed typical opioid activity *in vitro* and analgesic effects *in vivo*[9]. Met-enkephalin was recog-

H-Tyr-Gly-Gly-Phe-Met-OH [Ia] H-Tyr-Gly-Gly-Phe-Leu-OH [Ib]

nized as a part (residues 61-65) of mammalian and human β-lipotropin[10]. Larger sequences[11] and the entire COOH-terminal (61-91)-untriakontapeptide [II, β-endorphin] of camel pituitary β-lipotropin were found to exhibit potent opioid activity[12]. β-Lipotropin is thus another example of a "second order prohormone[13] i.e. a well-defined peptide hormone serving as a precursor for a smaller peptide with entirely different hormonal activities. The

H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-His-Lys-Lys-Gly-Gln-OH
65 70 75 80 85 90

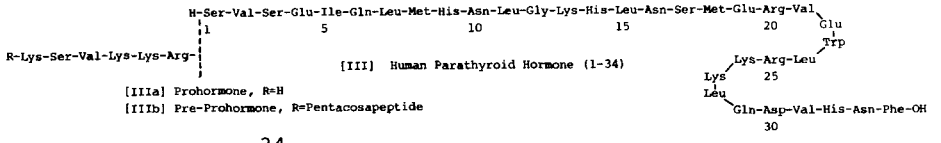
[II] β-Endorphin

characterization of the opioid peptides may suggest new approaches to the development of nonaddictive analgesics (see also Chapter 3).

Calcitropic (Thyroid) Hormones: Parathyrin, Calcitonin — Parathyrin (PTH) regulates calcium and phosphate levels in blood and influences bone metabolism. The chemistry, biology, and clinical utility of bovine and porcine PTH[5,14,15] have been reviewed. Sequence analysis of bPTH and pPTH showed these to be 84-residue linear peptides. Synthetic NH_2-terminal active

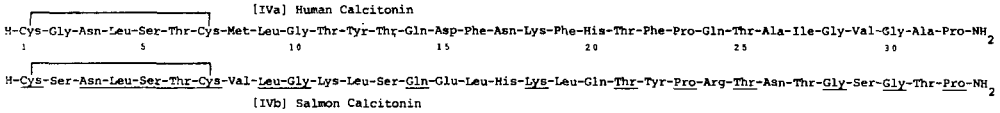
*Abbreviations: Small prefix letters denote species origin: b, bovine; h, human; p, porcine. A, Angiotensin; Acm, Acetamidomethyl; Boc, t-butyloxycarbonyl; Bpoc, 2-(p-Biphenylyl)isopropyloxycarbonyl; cAMP, Cyclic adenosine-3',5'-monophosphate; CCK, Cholecystokinin; CG, Choriogonadotropin; CNS, Central nervous system; CS, Chorioso**matoma**mmotropin; EGF, Epidermal growth factor; GH, Somatotropin (growth hormone); GI, Gastrointestinal; GIP, Gastric inhibitory peptide; IR, Immunoreactive; LH, Lutropin (luteinizing hormone); NGF, Nerve growth factor; Nle, L-norleucine; NSILA, Non-suppressible insulin-like activity; OBu^t, t-butyl ester; PTH, Parathyrin (parathyroid hormone); RIA, Radioimmunoassay; Trt, Triphenylmethyl; TSH, Thyrotropin; VIP, Vasoactive intestinal peptide.

cores, comprising residues 1-34, possess the full biological activities of the whole PTH. In addition to stimulating adenyl cyclase in bone and kidney cells, the synthetic peptides increased blood Ca^{++} and renal excretion of cAMP and phosphate in animals. The structure of the human hormone-(1-37) has been determined[16] and hPTH-(1-34) [III] was synthesized by the solid phase method[17]. On oxidative iodination for RIA purposes, the highly active



synthetic analog, [Tyr^{34}]bPTH-(1-34), suffered a severe loss of biological activity, due to methionine sulfoxide formation[18]. The sulfur-free synthetic analogs, [Nle^{8},Nle^{18}]bPTH-(1-34) and [Nle^{8},Nle^{18},Tyr^{34}]bPTH-(1-34), retained significant activity, which demonstrates that neither of the two Met residues is essential for biological activity. The latter analog was iodinated with nearly complete retention of its bioactivity[18]. — Biosynthesis of PTH proceeds via larger precursor molecules. bPTH prohormone [IIIa] contains a highly basic hexapeptide extension at its NH_2-terminal. In a still larger "pre-prohormone" [IIIb] the NH_2-terminal is further extended by 25 residues[19]. Increasing numbers of peptide hormone precursors have been recognized (review[20]) and it seems that hormone biosynthesis proceeds generally via precursors which subsequently undergo multistage enzymatic modification.

Calcitonin, the hypocalcemic, hypophosphatemic hormone originating from the thyroid gland, is the physiological antagonist of PTH. The structures of seven species-different calcitonins have been determined. Conventional syntheses of porcine, human [IVa] and salmon [IVb] calcitonin provided large supplies of homogeneous peptides for physiological, pharmacological, and



clinical investigations (review[21]). No active core has been detected; the entire molecule seems to be required for bioactivity. Salmon calcitonin is far more potent in man than human calcitonin, which may be attributable to increased metabolic stability. Since synthetic salmon calcitonin is now commercially available, chemical work on calcitonin has largely abated. Secretion of calcitonin from porcine thyroid glands is effected by activation of C-cell adenyl cyclase by either ionized Ca or by hormones (glucagon, gastrin, CCK, epinephrine). Calcitonin effects are age-dependent and are very low in normal adult humans. In young mammals calcitonin reduces bone resorption and plasma Ca^{++} and PO_4^{3+} concentrations by a net shift of Ca^{++} into bone[21]. — Medullary thyroid carcinoma is associated with elevated calcitonin concentrations in peripheral blood and may be diagnosed by calcitonin RIA[22]. The hormone has been successfully used in the clinical treatment of

Paget's disease[23].

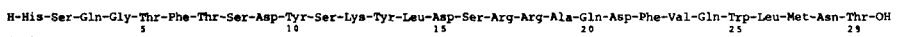
Thymic Hormones — Recognition of a thymus gland function in immune competence and evidence of an endocrine process have stimulated increasing efforts to identify and isolate individual factors[24,25]. Thymosin, isolated by a five-stage procedure from bovine thymus tissue[26] appeared to consist of several low molecular weight polypeptide components. This material was shown to enhance the one-way mixed lymphocyte reaction of mouse lymphoid cells[27] and was capable of immunologic restoration of both thymectomised and athymic nude mice[28]. In another approach, two closely related polypeptides, thymopoietin I and II, which induced the differentiation of early T-cells (thymocytes) from precursor prothymocytes[29], have been isolated from bovine thymus and their sequences [IV] of 49 amino acid residues been determined[30]. Thymopoietin action on T-cell precursors appears to be mediated by cyclic AMP[31]. A synthetic tridecapeptide corresponding to positions 29-41 of thymopoietin II exhibited 3% the activity of the native hormone in inducing the differentiation of T-lymphocytes[31]. Several other low molecular weight immune-stimulatory peptides have been isolated from thymus gland or blood plasma extracts[25]. The utility of thymic factors in the treatment of immune deficiency diseases and malignancies remains to be clinically confirmed.

Pancreatic Hormones: Insulin, Glucagon, Somatostatin — Eleven years after the initial successful syntheses of insulin, in which its two peptide chains were joined by random oxidative disulfide bond formation (review[32]), a genuine total synthesis of crystallized human insulin has been achieved[33,34] via consecutive formation of the correctly paired three disulfides[35,36]. In a fragment condensation approach[33,34], the amine component A(14-21)-B(17-30) [Va] containing the disulfide bridge between A20 and B19 was first condensed with fragment B(1-16) [Vb] followed by coupling with A(1-13) [Vc] in which the disulfide "loop" from A6 to A11 was already present. Finally the third disulfide bond between A7 and B7 was formed. This synthesis[33,34] represents a landmark in approaches to the total synthesis of natural peptides containing two or more disulfide bonds; however, it is unlikely to

become a commercial source of insulin in the foreseeable future, since natural resources appear to be sufficiently large. — The alternative of joining the two insulin chains by conformation-directed disulfide bond formation has been approached via (i) attempted proinsulin[20] synthesis[37,38] and (ii) reversible crosslinking of the A1 α-amino and the B29 ε-amino groups[39] which are in close iuxtaposition in the three-dimensional structure[40], or of the two α-amino functions[41] which are farther apart. — A reported[42] conversion of porcine to human insulin, by enzymatic removal of the B(23-30) octapeptide and replacement, after suitable protection, by the synthetic octapeptide of the human sequence, was not reproducible[43], as des-

cribed. In particular, the final saponification of insulin hexamethyl ester, produced biologically active mixtures of partially saponified hormone but no detectable native insulin[44]. The utility of conversion procedures employing enzymatic removal of the B(29-30) dipeptide[45] remains to be evaluated. — Syntheses of truncated insulins in search for an active core[43,46-48] established that only few amino acid residues may be deleted without substantial loss of activity. Des(B26-30)pentapeptide-insulin exhibited 35-45% the activity of the native hormone[46,48] but further deletions decreased the activity in agreement with recent insights into the receptor-binding region of insulin[49].

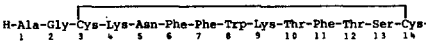
Glucagon [VI], has resisted repeated attempts at stepwise solid phase synthesis. A successful solid phase fragment condensation synthesis was reported[50]. The 1967 conventional synthesis of Wünsch *et al.* (review[51]) provided synthetic glucagon for biological studies. Glucagon sequences from human (identical with p and b hormones) and several other species have been



[VI] Human Glucagon

determined (review[52]) and a 37-residue "proglucagon" has been isolated and sequenced[53]. RIA studies revealed the presence of "enteroglucagon" or "gut glucagon" in human and animal intestine[4]. Secretory granules, indistinguishable from pancreatic α-cells, have been detected electronmicroscopically in canine gut[54], which could be the source of the extrapancreatic glucagon. — The recent discovery of the suppression of glucagon and insulin secretion in animals and in man by somatostatin (see also Chapter 18) stimulated several studies on blood glucose regulation. It appears that glucagon might play a fundamental role in maintaining blood glucose levels, while insulin is important in minute to minute regulation[55]. Glucagon with its gluconeogenic, ketogenic, and lipolytic action may be as responsible for the development of acute diabetic ketoacidosis and the full hyperglycymic syndrom as low insulin or decreased responsiveness to insulin[54,56].

Somatostatin [VII], its chemistry and biology, its presence in spinal cord and various areas of the brain and in gastrointestinal organs, and its inhibitory influence on the secretion of GH, TSH, gastrin, glucagon and in-

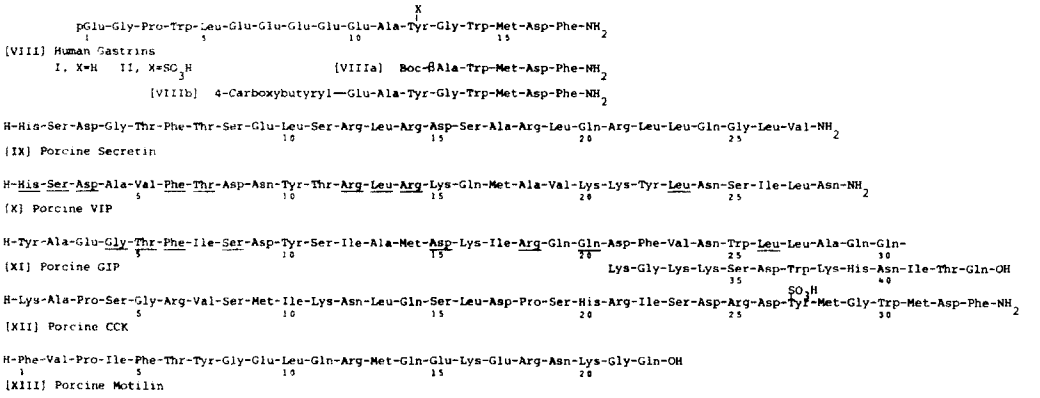


[VII] Somatostatin

sulin, have been reviewed[57] (see also Chapter 18). An RIA was developed[58] using a rabbit antiserum against iodinated [Tyr^1]somatostatin conjugated with serum α-globulin. No circulating VII has yet been detected, and its presence in pancreas[59], stomach, duodenum, and jejunum suggests sites of production in addition to the hypothalamus. Synthesis[57,60] aims at long-acting analogs with increased resistance to enzymatic degradation and selective suppression of glucagon release. A nonreducible cyclic des-(Ala^1-Gly^2)-dicarba analog with prolonged action[61], an orally active derivative (N-propionyl-des-Ala^1-somatostatin)[62], and a shift of the pharmacological activity profile toward selective insulin suppression with des-(Ala^1-Gly^2)dihydrosomatostatin[63] have been discovered. — Enzymatic inactivation of somatosta-

tin by brain extracts proceeds via cleavage of the Trp-8 to Lys-9 bond[64]. Synthetic [D-Trp^8]somatostatin exhibited 8 times the activity of the native hormone[65]. — Besides potential therapeutic utility for the control of diabetes mellitus as an adjunct to insulin[56], appropriate somatostatin analogs may also have potential use for the treatment of peptic ulcers[66] in view of the recent findings that somatostatin inhibits gastrin release[67].

Gastrointestinal Hormones — At least 11 different types of endocrine cells have been identified in the gastrointestinal mucosa by electron microscopy[68,69]. Since these cells do not occur in cumulative glandular form but are widely scattered throughout the gut, identification and isolation of their respective hormones is much more difficult than with glandular hormones[70]. Thus far, the structures of six GI hormones have been elucidated, i.e. gastrin [VIII], secretin [IX], vasoactive intestinal peptide (VIP) [X][71], gastric inhibitory peptide (GIP) [XI], cholecystokinin/pancreozymin (CCK) [XII], and motilin [XIII]. These range in size from the 17-residue



gastrin (mol wt 2200) to the 43-residue GIP (mol wt 5105) and encompass very acidic (gastrin), neutral (motilin), and very basic (secretin) peptides. RIAs have been developed for each[4]. Presumably, these peptides originate from larger biosynthetic precursors (prohormones), but none has been described yet. — Gastrin regulates and induces gastric acid secretion. The duodenal hormones [XIII-XVI] are involved in neutralization of gastric acid by release of pancreatic bicarbonate (secretin, VIP), in enzyme and gall bladder stimulation (CCK), in inhibition of gastric acid secretion (GIP), in promotion of vasodilation and hypotension (VIP), in stimulation of gut mucosal secretion (secretin, GIP, VIP), and in promotion of gastric motility (motilin). — A particular feature of the GI hormones are their overlapping or identical actions[69,71]. For example secretin, GIP, and VIP all inhibit gastric acid secretion; gastrin and CCK stimulate gastric acid and pancreatic enzyme production; and motilin and CCK have powerful gut motor activity. This appears to be interrelated with certain structural similarities[5]. Statistically significant sequence homology exists between secretin, GIP, and

VIP (and glucagon) and evolution from a common ancestral protein has been suggested[72]. Similarly, gastrin and CCK have a common COOH-terminal tetrapeptide sequence. Motilin, however, possesses a unique sequence unrelated to any of the other GI hormones. Several comprehensive reviews on the chemistry and biology of the GI hormones have appeared[5,73-77].

Gastrin [VIII] (review[78]) has the smallest highly active core of all known peptide hormones. The COOH-terminal tetrapeptide exhibits 23% of the secretagogue activity of VIII. Several analogs with higher potency than the tetrapeptide core have been found, of which pentagastrin [VIIIa][79] is used in clinical diagnosis of gastric secretion. However, a desired potent inhibitor of the hormone has not been discovered among ca. 500 analogs synthesized[80]. It has been reported that pentagastrin [VIIIa], but not the native hormone [VIII], is inactivated by passage through the liver[81]; therefore, some compounds of potential interest might have escaped detection by confining most of the analog program to small peptides. Indeed, elongation at the NH_2-terminal resulted in hepta- and octapeptides [VIIIb] with increased potency[82]. Structure—activity relations of the smaller synthetic analogs have been discussed[80,83]. — The variety of circulating forms of gastrin, smaller and larger than the native hormone [VIII], which are detectable by RIA[4], have been discussed[75]. Clarification of the relationship between the biological and immunochemical properties of the various gastrins and their endocrinologic significance will depend on full characterization of each component. The preparation of pure human "big" gastrin (G34) has been reported[84]. — It is difficult to differentiate physiological from pharmacological effects. The primary function of the hormone may well be its trophic action[78], which maintains the functional integrity of GI tract tissues. The antagonistic effects on gastric acid secretion elicited by secretin, VIP, and GIP are part of the complex and subtle interplay of the GI hormones[85].

Secretin [IX] was first synthesized by conventional methodology using both stepwise addition of amino acids[86] and fragment condensation[51,86], which provided products possessing the same potency (4000 U/kg) and the same spectrum of biological activity as purified natural porcine secretin. Lower potency and less pure material was obtained by solid phase synthesis . Recent preparative scale syntheses of secretin have been carried out by advanced conventional methodology[88,89]. It appears that the entire secretin molecule is required for biological activity and for assuming its partially helical folded conformation[86], since even removal of a single residue at the NH_2-terminal caused inactivation. — Secretin is the most potent known stimulant of pancreatic bicarbonate secretion (reviews[74,75,90]). A role of cAMP in secretin action has been suggested[91]. The circulation half-life of secretin in dogs is 3.2 min; enzymatic degradation occurs in plasma and during liver passage. By comparison, stimulation of pancreatic HCO_3^- secretion in dogs by VIP was 17% that of secretin, indicating that both hormones can interact with each other's receptor sites[92]. In birds, stimulation of pancreatic secretion by porcine secretin was weak[93]. Conversely, porcine VIP showed potent activity in birds but was weak in mammals. Extracts of chicken or teleost fish intes-

tine exhibited weak secretin-like effects on the mammalian pancreas, but strong effects on the pancreas of birds, suggesting a long evolutionary history of these hormones[93]. The recent discovery that secretin stimulates insulin release from pancreatic islet cells opened up an active field of investigation[94,95]. Other actions of secretin include stimulation of gastric pepsin secretion and increase in mesenteric blood flow. Secretin-induced mesenteric vasodilatation may, in part, be due to a direct effect of the hormone on vascular smooth muscle[96]. — Clinical use is made of secretin as a diagnostic aid for chronic pancreatitis or cancer of the pancreas, and in hypotonic duodenography[97].

VIP [X] is a highly basic octacosapeptide the structure of which[76,98] has been confirmed by total synthesis[99]. The sequence of chicken VIP[100] differs from [X] in four positions. The entire molecule is required for full biological activity, smaller COOH-terminal fragments exhibit lower potency. VIP exhibits potent vasodilator and hypotensive actions in peripheral, splanchnic, coronary, and pulmonary vascular beds, and a wide range of other actions on stomach, pancreas, gall bladder, intestine, and the respiratory system (review[76,98]).

GIP [XI] inhibits secretion of gastric acid and pepsin[76,101], stimulates insulin release[102] and might have a role in carbohydrate metabolism in man. It has no effect on secretion of the exocrine pancreas[93]. The entire tritetracontapeptide [XI] has recently been synthesized by conventional solution methodology and exhibited full GIP activity[103].

CCK [XII] exhibits dual effects on gall bladder contraction and pancreatic enzyme secretion and was long thought to be a mixture of two hormones (cholecystokinin and pancreazymin) until Jorpes and Mutt[104] decided the issue in favor of a single hormone by exceedingly thorough purification and sequence analysis. The COOH-terminal (29-33) pentapeptide amide is identical with that of gastrin [VIII]. Parts of the molecule showed higher activity than intact XII. The COOH-terminal (22-33)-dodecapeptide amide [XIIa] was four times, the (26-33)-octapeptide amide [XIIb] 10 times as active as CCK. Syntheses have been reported for the NH_2-terminal hexa- and octapeptides (inactive)[105], for XIIa and XIIb[106] and for several other analogs[107]. The entire molecule [XII] has not yet been synthesized. — The biological properties of CCK have been reviewed[74,98,108].

Motilin [XIII], the latest GI hormone of known structure[109], has been isolated from the mucosa of the small intestine. The 22-residue porcine peptide was discovered by its potent (100 ng/kg) motor stimulatory effects on fundic and antral pouches of the dog stomach. The analog, [13-norleucine, 14-glutamic acid]-motilin, was first synthesized[110] and was nearly as active as natural motilin. The synthesis of XIII has been accomplished recently[111] by conventional methodology in solution via four intermediate protected peptides. The azide procedure in the Honzl-Rudinger variant was used for all but one peptide bond condensation to minimize the risk of racemization. The synthetic product possessed full biological activity. In another synthetic approach, solid phase methodology and new coupling

reagents, diphenyl phosphorazidate and diethyl phosphorocyanidate, were utilized[112]. The biological activity profile of synthetic [Nle^{13},Glu^{14}] motilin in animal and human gastric mucosa, smooth muscle and GI tract preparations, in enzyme stimulation and in protein synthesis was studied in detail[113]. — An RIA with a sensitivity range of 10-320 pg has been developed[114], using antisera to porcine motilin in guinea pigs, and highly purified ^{125}I-motilin as a tracer. No cross-reactivity with any other GI hormone was detected. Strong evidence for motilin being the peptide hormone released upon alkalinization of the duodenum was obtained by experiments in dogs that resulted in simultaneous and parallel increases of both circulating levels of IR-motilin and fundic motor pouch activity in response to alkalinization challenge[114]. The motilin endocrine cells have been fully identified in human and animal duodenal and jejunal mucosa by immunofluorescent techniques[115].

Chorionic (Placental) Hormones — Human choriogonadotropin (hCG) is produced by the placenta predominantly in early pregnancy, human choriosomatomammotropin (CS, also called "placental lactogen") in late pregnancy. The glycoprotein hCG, composed of two subunits (α and β)[116,117] is structurally and functionally related to human lutropin (hLH)[118]. The single-chain hCS[119,120] exhibits lactogenic and somatotrophic activity similar to human somatotropin (hGH)[121] to which it bears extensive (83%) structural homology. Structure elucidation of hCG[122,123] showed almost full homology of the α-subunit (92 residues) with hLHα which differs only in a 2-residue inversion and a 3-residue deletion at the NH_2-terminus. Indeed, the α-subunits of hCG, LH, and TSH are interchangeable and the resulting reconstituted hormones are indistinguishable from the native hormones in biological, immunological, and electrophoretic properties[122]. The β-subunits determine hormone specificity. The carbohydrate structures of both subunits of hCG differ from those of LH and FSH[116,122]. hCGβ contains a COOH-terminal sequence of 30 residues which does not occur in hLH and offers preparation of hCG-specific antibodies. A derivative of the hCGβ-(125-143)-eicosapeptide has been synthesized, by conventional solution methods. It induced antibodies to the entire hormone[124] and may offer diagnostic potential for early detection of hCG-producing tumors.

Ovarian Hormone — Relaxin is responsible for the widening of the cervix and the suppression of uterine motility in mammals. It has recently been detected in human pregnancy serum by RIA[125]. Relaxin, which has mol wt 5,600, consists of a 22-residue A chain and a 30-residue B chain linked by disulfide bonds similar to insulin[126,127]. The A chain sequence[127] shows no homology with the A chain of insulin, except for the distribution of the half-cystine residues. Selective NBS oxidation of one of the two Trp residues of the B chain did not affect hormonal activity, but complete oxidation decreased it[128].

Vasoactive Tissue Hormones: Angiotensins, Kinins, Substance P, Neurotensin — Comprehensive books have appeared on the chemistry and biology of angiotensins I and II [XIVa,b][129 130] and on bradykinin [XVa] and related peptides[131 132]. AII regulates blood pressure, water balance, and salt metabolism[133], and aldosterone release from the adrenal cortex. (Des-Asp^1)-AII may also be an important agonist ("angiotensin III")[134], especially in aldosterone release. Several hundred analogs have been synthesized[129 130] including several highly active competitive inhibitors, among which [1-sarcosine, 8-L-O-methylthreonine]AII is so far the most potent[135].

Research on mammalian kinins has been reviewed[136]. The over 30 natural kinins of known structure comprise (i) physiologically significant mammalian tissue hormones [XVa-f] and (ii) inflammatory venoms of stinging insects,

Table I. Structures of Selected Kinins and Release of Mammalian Kinins from Precursor Plasma Protein

Homologies			
	...Gly-Arg-Met-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg...		Bradykininogen
	Plasma-Kallikrein, Trypsin	[XVa]	Bradykinin
	Glandular-Kallikrein	[XVb]	Kallidin
	Plasmin	[XVc]	ML-Bradykinin
	Pepsin-like Protease	[XVd]	GAML-Bradykinin
	H-Thr-Ala-Thr-Thr-Arg-Arg-Arg-Gly-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH	[XVIa][a,b]	Vespulakinin 1
	H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2	[XVe]	Substance P
	pGlu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH_2	[XVIb][c]	Physalaemin
	pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH	[XVf]	Neurotensin
	pGlu-Gly-Lys-Arg-Pro-Trp-Ile-Leu-OH	[XVIc]	Xenopsin
	pGlu-Gln-Asp-Tyr(SO_3H)-Thr-Gly-Trp-Met-Asp-Phe-NH_2	[XVId][d]	Caerulein
	pGlu-Val-Pro-Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH_2	[XVIe]	Ranatensin
	pGlu-Gln-Arg-Leu-Gly-Thr-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2	[XVIf]	Alytesin
	pGlu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2	[XVIg]	Bombesin
	pGlu-Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH_2	[XVIh]	Litorin

[a] $CH_{1,2}$ = 1-3 N-Ac-galactosamine, 1-2 galactose. [b] Related: Polisteskinin, Wasp Kinin. [c] Related: Eledoisin, Phyllomedusin. [d] Compare with Gastrin (VIII). Related: Phyllocaerulein.

amphibians, and mollusca [XVIa-h]. Striking sequence homologies (Table I) exist between bradykinin [XVa] and vespulakinin [XVIa][137] (and also phyllokinins and other COOH-terminally extended kinins), between substance P[XVe][1] and physalaemin [XVIb], between neurotensin [XVf][1] and xenopsin [XVIc][138], between gastrin [VIII] and caerulein [XVId], and among the venoms ranatensin [XVIe], alytesin [XVIf], bombesin [XVIg] and litorin [XVIh][139]. Caerulein [XVId], with a COOH-terminal pentapeptide identical to that of both gastrin [VIII] and CCK [XII], is more potent than gastrin in stimulating gastric acid secretion in dogs[140]. Physiologically, mammalian kinins may be involved in the regulation of blood pressure[141], capillary permeability, and blood clotting[136], and in anaphylactic and traumatic shock, in allergic and rheumatic disorders[131], and in the generation of pain. Neural control of kinin action has been suggested[131,142]. The clinical treatment of kinin-

related disorders has been reviewed[132]. Vespulakinins [XVIa], recently isolated from the venom of the yellow jacket[137], are the first reported naturally occurring glycopeptide derivatives of bradykinin and the first reported vasoactive glycopeptides.

Growth Factors: Somatomedins, Epidermal (EGF) and Nerve Growth Factor (NGF), Urogastron (UG) — Somatomedins[1] (review[143]) are plasma factors, of uncertain origin, in man and animals. High-affinity binding sites have been found in a variety of mammalian tissues, such as cartilage, liver, and muscle. Their biological significance as growth-promoting agents has not yet been established. These factors have weak insulin-like activity. However, their ability to promote anabolic processes in various cells in tissue culture has been found recently to be two orders of magnitude greater than the insulin-like effects[143]. NSILA-S is chemically best characterized and appears to be a single-chain peptide of mol wt ca 6000 containing 3 disulfide bonds[144].

EGF, a single-chain 53-residue peptide of known structure (mol wt 6045), isolated from adult male mouse submaxillary glands (review[145]), stimulates proliferation and keratinization of epidermal tissue and is a potent mitogen in human or mouse fibroblasts in culture. Human EGF, isolated from urine, is somewhat smaller (mol wt 5300-5500) but of similar biological activity[146]. The physiological role of EGF is unclear. Inhibition of pentagastrin- or histamine-stimulated gastric acid secretion in dogs by EGF has recently been described[147].

Two urogastrons (β-UG, γ-UG), differing in one COOH-terminal Arg residue, have been isolated from human urine, and their structures determined as 53(52)-residue single-chain peptides with 3 disulfide bonds[148]. They are similar to murine EGF both chemically, with a sequence homology of 37 common residues, and biologically in stimulating epithelical cell tissue growth and in inhibiting pentagastrin- or histamine-stimulated gastric acid secretion specifically, i.e. without effecting other secretions such as pancreatic, biliary or salivary secretion. It has been suggested[148] that urogastron might be identical with hEGF[146,147].

Murine NGF consists of two identical non-covalently linked subunits of 118 residues (mol wt 13,259) each, of known structure (review[149]). The mode of action of NGF in development and maintenance of sympathetic neurons and central neuronal connections and in regenerative processes has been reviewed[150]. The biosynthesis of NGF may proceed via larger precursors[151]. NGFs, isolated from mouse salivary glands or from snake venoms[152], have been compared with insulin structurally and biologically, and certain similarities have been observed, which led to the proposal[149] that these peptides might be derived from a common, albeit distant, ancestral protein.

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Chapter 18. Diabetes Mellitus

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The bihormonal-imbalance hypothesis of diabetes mellitus[1] has gathered considerable momentum[2] since 1974 when this subject was reviewed here last[3]. The hypothesis depicts that a combination of insulin deficiency and glucagon overabundance leads to abnormal glucose metabolism characteristic of diabetes mellitus. Consequently, the ideal treatment for diabetic patients would be a combined therapy of stimulating insulin release and inhibiting glucagon secretion. Somatostatin, a third hormone in the pancreatic islets[4-6], was discovered to be a potent inhibitor of glucagon and insulin release[7-9] and it may prove to be a useful adjunct to insulin in treating diabetes mellitus[10,11].

This review covers selected papers published since 1974 on these three endocrine factors, and it also updates the literature on diabetic microangiopathy and oral hypoglycemic agents.

Insulin

Secretion. Three models have been suggested for the stimulation of insulin release by its primary secretagogue - glucose. The "regulator-site" hypothesis[12] favors the notion that glucose directly interacts with a receptor in the cell membrane leading to an excitation of the insulin secretory process. Several observations during the past two years lend support to this view. Niki et al.[13] first reported that the α-anomer of D-glucose is more effective in stimulating insulin release. Subsequently, other investigators confirmed this finding and additionally found the α-anomer to be more potent in inhibiting glucagon release in the islet α-cells[14,15]. However, the α-anomer serves no more effectively than the β-anomer as a substrate for various metabolic activities of the islet cell[16]. These reports strongly support the existence of a glucoreceptor in islet cells with differential reactivity to the anomers of glucose. An additional line of evidence came from the discovery that a sugar, digitoxose, inhibits glucose-dependent insulin release but not glucose oxidation in the islets[17]. Based on some of these findings, as well as the report that diabetic patients showed impaired ability to taste glucose but not fructose[18], Niki and Niki suggested that diabetes mellitus may arise from a "generalized disorder of a glucoreceptor"[19]. However, as attractive as this hypothesis may be, its simplistic view is unable to explain the abnormal response of insulin release to a variety of substances as observed in animals with spontaneous diabetes[20,21].

In the second model, i.e. the "substrate-site" hypothesis[22], it is assumed that the stimulus comes from a metabolite of glucose and that metabolism of glucose in the β-cell is essential for insulin release. Support for this model comes from studies on insulin release in vitro in the presence of various glucose analogues and metabolites[23-25]. In particular, two trioses in the glycolytic pathway, D-glyceraldehyde and

dihydroxyacetone, were found to be potent insulin secretagogues[24-26]. Their insulinotropic actions were not affected by D-mannoheptulose which is a strong inhibitor for glucose-triggered insulin release. Although the findings do not provide conclusive evidence to eliminate the existence of an islet cell glucoreceptor, the results do suggest the complex nature of the β-cell secretagogue recognition site which interacts with glucose, its analogues and metabolites with differential sensitivity. These investigations also point to the likelihood of a third model in β-cell recognition of its secretagogues, namely a "two-site" model[23]. The model consists of an "initiator" site which recognizes glucose and its metabolizable analogues and a "potentiator" site which has even broader specificity and binds those sugars which are poorly metabolized or not metabolized at all by the β-cells. Binding at the "potentiator" site will elicit insulin release provided that the "initiator" site is also activated. The observation that sugars which alone are ineffective in stimulating insulin release will show insulinotropic activity when glucose is also present at a substimulatory concentration[27] lend credence to the "two-site" model. Sugars with "potentiator" activity include D-fructose, N-acetylglucosamine, D-galactose, and L-glyceraldehyde[23,27].

The event immediately following the initial signal exerted by the secretagogues still remains to be unravelled. Although it has been reported that glucose increased cAMP concentration in perifused islets[28], studies on the effects of cAMP and agents that increase its intracellular levels in islets in vitro yielded discrepant data between cAMP-promoting and insulin-releasing activities[29,30]. These findings suggest that glucose does not initiate insulin release by increasing cAMP levels and that cAMP acts primarily as a positive modulator of glucose-induced insulin release. An evaluation of intracellular Ca^{++} concentration appears to be an essential step between β-cell recognition of glucose and the eventual release of insulin[31-33]. Although the sequence of biochemical events between these two steps is largely unknown at this moment, detailed studies of the morphology of pancreatic β-cells revealed the involvement of microtubules and microfilamentous cell webs in the translocation and exocytosis of the secretory granules[34,35].

Although it has been reported that subjects predisposed to the development of diabetes showed impaired insulin response to oral glucose[36], conflicting data have also been obtained[37,38]. In two studies with monozygotic twin siblings of diabetic patients, the authors found no significant difference in glucose-insulin relationship between normoglycemic monozygotic twins of diabetic patients and control subjects matched for age and sex[39,40]. In a retrospective study on subjects who subsequently developed diabetes, these "true prediabetics" were found to have had neither excessive nor diminished insulin secretion in comparison with controls who remained normal during follow-up[41].

Receptor. The concept that insulin exerts its biological activities by interaction with the plasma membrane has been strengthened by the demonstration of insulin receptors in adipocyte plasma membranes by means of a ferritin-insulin conjugate[42]. Using electron microscopy, ferritin-

insulin was found exclusively on the concave side of surface-connected vesicles in the plasma membrane of isolated adipocytes[43]. The binding of insulin to its receptors induced negatively cooperative interactions among binding sites resulting in curvilinear Scatchard plots[44]. Kinetic studies of insulin-receptor interactions and insulin-induced lipogenesis in isolated adipocytes revealed a more complex relationship than direct proportionality between initial binding and resulting biological activity[45].

The involvement of insulin receptor changes in the pathological state of obese-hyperglycemic animals has been firmly established. In at least three strains of mice with hereditary obesity, hyperinsulinemia and hyperglycemia at certain stages of their lives, a decrease in the absolute number of insulin receptor molecules correlated well with the apparent insulin resistance which these animals exhibited[46-48]. The loss in insulin receptors appeared to be the result of a self-regulatory process by which the biological effects of insulin are modulated in vivo. Evidence to reinforce this notion arises from the observation that a reduction in hyperinsulinemia by food restriction in obese mice led to an increase in insulin receptor concentration and that maintaining hyperinsulinemia in these fasting animals by administering exogenous insulin prevented this increase[46]. Investigations along these lines have been extended to human subjects with similar results[49,50]. In addition, increased insulin binding capacity was found in liver plasma membranes of two types of insulin-deficient animals, the diabetic Chinese hamsters[51] and streptozotocin-treated rats[52]. These findings lend credence to the proposition that the prevailing insulin level may modulate its receptor concentration.

Mechanism of Action. Papers on the multitudinous biological effects of insulin continue to accumulate. A review article on the significance of cAMP-dependent protein kinases as mediators in the action of insulin on cell metabolism has appeared[53]. Another hypothesis suggests that insulin acts mainly by redistributing intra- and extracellular Ca^{++} concentration, a rise in intracellular Ca^{++} constituting a common signal for the initiation of cellular activities modulated by insulin[54,55]. A third view favors the theory that insulin acts by connecting hexokinase to mitochondria where hexokinase-generated ADP serves as a stimulus-substrate for further production of ATP to be used in the anabolic reactions[56]. Although reports on insulin-mediated changes in cAMP-dependent protein kinases, intracellular Ca^{++} concentration and hexokinase activity have been amply cited in review articles, all three concepts remain to be substantiated experimentally.

Glucagon

The opposing biological effects of glucagon and insulin have long been implicated in the pathogenesis of diabetes mellitus. The incongruity between the fact that glucagon is produced in pancreatic islet α-cells and the observation that total pancreatectomy induces severe hyperglycemia argues against this hypothesis. However, several groups of investigators have reported significant levels of glucagon present in the plasma of totally pancreatectomized dogs[57-59]. The extrapancreatic glucagon is of

gastrointestinal origin. Extracts of porcine duodenal mucosa have been found to contain a protein fraction with chromatographic and immunologic properties similar to pancreatic glucagon[60]. Furthermore, electron microscopic observations revealed cells in the canine gastric fundus with secretory granules indistinguishable from pancreatic α-cells[61]. Thus surgical removal of the pancreas does not result in a combined deficiency of insulin and glucagon as assumed previously.

The second line of evidence pointing to the significance of hyperglucagonemia in diabetes comes from reports of excessive glucagon secretion in all forms of diabetes, including spontaneous diabetes in both man[62] and animal[21] and experimental diabetes produced by alloxan[63], streptozotocin[64], mannoheptulose[63], anti-insulin serum[63], and diazoxide[63,65]. Even more conclusive evidence that glucagon overproduction contributes to severe hyperglycemia was the appearance of hypoglycemia in animals infused with somatostatin which inhibits both insulin and glucagon secretion[65]. Results from a similar study in human diabetic subjects by Gerich *et al.* indicate that hyperglucagonemia accounts for about 25% of the fasting plasma glucose levels[10]. The same group of investigators studied the effect of glucagon levels on changes in plasma β-hydroxybutyrate and glucose concentrations after acute withdrawal of insulin in juvenile-onset diabetic patients[67]. They observed marked reduction in plasma β-hydroxybutyrate, glucose, free fatty acid, and glycerol levels during somatostatin-induced suppression of glucagon secretion and concluded that glucagon is essential for the development of ketoacidosis in man. It therefore appears that diabetic abnormalities in glucose homeostasis and lipid metabolism are the combined consequence of insulin deficiency (leading to underutilization of glucose) and glucagon overabundance (leading to excessive gluconeogenesis, lipolysis, and ketogenesis).

Renewed interest in the diabetogenic role of glucagon has prompted intensive study of the mechanism of glucagon biosynthesis and secretion. Like insulin, glucagon is produced via a larger precursor molecule[68]. Normally, its secretion is inhibited by glucose, glyceraldehyde, and insulin[69] and stimulated by amino acids[70]. In diabetes, however, hyperglycemia fails to inhibit oversecretion of glucagon[21,62-65] and this phenomenon suggests that glucose-induced suppression of glucagon release may be insulin-dependent. On the other hand, in a study employing isolated pancreas of alloxan- and streptozotocin-diabetic rats, Pagliara *et al.* observed potent inhibition of amino acid-induced glucagon suppression by glucose, which suggests that extrapancreatic factors are responsible for the hyperglucagonemia in the diabetic animals[70]. A recent study showed that glucose stimulates glucagon release in perfused rat pancreas *in vitro* during Ca^{++} deprivation[71]. This finding points to the importance of Ca^{++} in regulating both insulin and glucagon secretion and its possible role in the pathogenesis of diabetes mellitus.

Somatostatin

Somatostatin, a tetradecapeptide, was first isolated from sheep hypothalamus and was found to be a potent inhibitor of the release of pituitary

growth hormone, somatotropin, from which its name is derived[72]. It is also known as SRIF (somatotropin release inhibiting factor) and GRIH (growth hormone release inhibiting hormone). It was discovered later to suppress the secretion of thyrotropin, prolactin[73,74], gastrin[75], and insulin[76] as well, although only at relatively high concentrations. Since hypothalamic hormones are present at extremely low levels in peripheral blood[77], the requirement of disproportionately large doses of somatostatin to exert an action on gastrin and pancreatic hormone release suggested that it may also be present in gastrointestinal organs. Indeed, significant levels of somatostatin were found in stomach, pancreas, duodenum and jejunum[78]. The islet D cell has been identified as the site of somatostatin production in the pancreas by means of immunofluorescence[5].

The observation by Koerker et al. that plasma glucose levels fall during somatostatin infusion in baboons led to the discovery that somatostatin is a potent inhibitor of both insulin and glucagon secretion[66]. A number of investigators have extended these studies to man and confirmed the action of somatostatin on the endocrine pancreas and its potential as a useful adjunct to insulin in treating diabetes[10,67,79,81]. However, its short duration of action, its inhibitory effect on multiple endocrine factors, as well as its undesirable side effects such as dizziness, nausea, and impairment of platelet function[82], appear to limit its use as a therapeutic agent. Accordingly, an intensive search for synthetic analogues with prolonged and specific action is underway in several laboratories. Several more potent analogues have been reported[83,84] but appear to offer no potential therapeutic advantage[85].

Diabetic Microangiopathy

Reviews on the various aspects of diabetic microangiopathy have been published[86,87]. Deterioration of the microcirculation in diabetes is often observed in such body areas as retina, kidney, peripheral nerve, extremities, heart, and arteries. The search for an understanding of the sequence of events leading to the development of diabetic microangiopathy has been intensive but a unifying theory is still lacking. Major efforts have been made to elucidate the biochemical abnormalities associated with capillary basement membrane thickening which is observed in diabetic patients. Although the hypothesis of Spiro et al. that the change in diabetic glomerular basement membrane is due to increased activity of glycosyltransferases and an increased content of hydroxylysine-linked glucosylgalactosyl disaccharide units[88] remains attractive, there are conflicting reports indicating that hydroxylysine content in the glomerular basement membrane of diabetic subjects[89,90] and kidney glycosyltransferase activity of the diabetic mice[91] are not elevated.

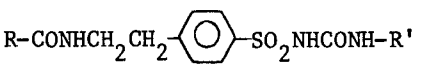
Two biochemical entities known to affect tissue oxygen delivery have been reported to be abnormal in diabetic patients. Increased levels of glycosylated hemoglobin Alc which has high affinity for oxygen have been shown to be present in both human diabetics[92,93] and hereditary diabetic mice[94]. Also, a decrease in 2,3-diphosphoglycerate content, which is closely related to the function of hemoglobin, has been observed to occur

in erythrocytes of diabetics in ketoacidosis[95]. This suggests that an underlying cause of diabetic microangiopathy may be hypoxia which could arise from the decreased ability of the circulating blood of diabetics to deliver oxygen to the appropriate tissue sites[96,97].

Whether proper long-term control of blood glucose allevaites or reverses diabetic complications has long been questioned. The results from two recent studies provide a positive answer to this question. Prevention and regression of diabetic microvascular lesions were observed in diabetic rats after pancreas[98] or islet transplantation[99]. Organ transplantation or implanted artificial β-cells with finely controlled insulin release mechanism therefore appear to offer the potential of being superior to conventional insulin therapy. Several laboratories are engaged in exploring the feasibility of employing these techniques in humans but their practicality needs to be established[100-102].

Oral Hypoglycemic Agents

Several new sulfonylureas have been reported to be highly potent oral hypoglycemic agents. Glypentide (I)[103], gliflurmide[104] (glioptamide[105], II) were about 1000 fold more potent than tolbutamide and their action is longer lasting. Glicaramide (SQ65993, III) was shown to decrease blood glucose concentration in normal and experimental diabetic animals and to lower plasma triglyceride and free fatty acid levels[106]. Two other hypoglycemic sulfonylureas, glisoxepide (RP-22410 IV)[107] and glipizide (K-4024 V)[108], were shown to inhibit gluconeogenesis from L-lactate and L-alanine in rat liver in vitro. Clinical and pharmacokinetic studies of glipizide demonstrated that it was rapidly absorbed and highly effective in maturity-onset diabetic patients[109-111]. Sulfonylureas appear to have no direct effect on glucagon release by the pancreatic α cells [112,113]. However, long-term administration of sulfonylureas may reduce the hyperresponsiveness of arginine-induced glucagon release in diabetic patients[114].



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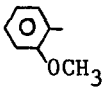
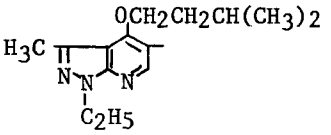
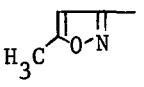
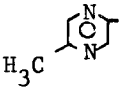
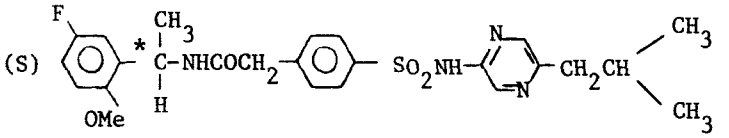
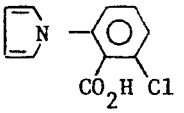
I		
III		
IV		
V		



(II)

Dichloroacetate induced hypoglycemia in normal and diabetic rats[115, 116], and it significantly decreased ketone body levels in blood when infused alone or in combination with insulin[117,118]. It also inhibits hepatic gluconeogenesis and decreases hepatic glycolytic enzymes[119]. The hypoglycemic effect of T-9878 (VI) was attributed to a stimulatory action on glucose utilization[120]. A number of other 1-(2-carboxy-phenyl)-pyrroles were also found to lower blood glucose[121]. Other classes of compounds reported to have oral hypoglycemic activity include 2-(N-Alkylaminomethyl) indolizines[122] and N-substituted indanamides[123].



(VI)

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Chapter 19. Disorders of Lipid Metabolism: Etiology and Therapy

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Introduction - This review will highlight the recent literature on lipoprotein metabolism, the relationship of low density lipoprotein receptors and lipoprotein lipase to atherogenesis, and the relevance of insulin and glucagon to hyperlipidemia.[1] Novel and established lipid lowering drugs will be discussed. The literature on the pathogenesis of gallstones and their dissolution by chenodeoxycholic acid will be reviewed.

Regulation of Cholesterol Metabolism in Normal and Abnormal States - Several reviews relating lipoprotein structure to function and metabolism have appeared.[2,3] All five lipoprotein families, chylomicrons, very low density lipoproteins (VLDL), intermediate lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) are linked metabolically as follows: 1) VLDL and chylomicrons, the chief triglyceride transporting particles, carry triglycerides from synthetic sites in liver and intestine to utilization sites in muscle and adipose tissue, where extrahepatic lipoprotein lipase hydrolyzes the triglycerides with concomitant transfer of apoprotein C subunits to HDL and conversion of apoprotein B subunits to IDL; 2) IDL may be catabolized by hepatic lipoprotein lipase to LDL; 3) HDL and LDL are removed from the circulation by tissues. An elegant computer model for human lipoprotein metabolism has been developed.[4]

Cholesterol is absorbed from the intestine and appears in chylomicrons and VLDL as cholesterol esters. Hepatic removal appears to be the major catabolic pathway for cholesterol esters of VLDL.[5] When the secretion of rat VLDL into plasma was blocked by 4-amino-pyrazolo-pyrimidine, triglycerides from chylomicrons and VLDL were metabolized rapidly, whereas most of the cholesterol esters remained in a less dense small remnant lipoprotein fraction.[6]

Factors regulating cholesterol synthesis in hepatocytes have been elucidated which suggest that the relative rates of cholesterol efflux and influx regulate 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity.[7,8] Hepatic cholesterol synthesis was modulated in vivo by bolus injection or continuous infusion of various lipoproteins.[9,10] Only rat liver demonstrated net cholesterol uptake from circulating lipoproteins and only this tissue manifested inhibition of cholesterogenesis which was related to the incremental increase in cholesterol esters. These studies emphasized the primary role of cholesterol carried on lipoproteins of intestinal origin, since the greatest inhibition of cholesterol synthesis occurred with intestinal lipoproteins having Sf values >8000. The depression in endogenous synthesis by dietary cholesterol was made up precisely

by the amounts of cholesterol derived from the diet, thus supporting the hypothesis that exogenous cholesterol and synthesized cholesterol enter the same hepatic pool.[11]

Although LDL degradation is generally considered a hepatic process involving hepatic lipase, conflicting data exist. The half-life of LDL in swine decreased rather than increased after functional hepatectomy, suggesting that non-hepatic tissues may constitute a major site for LDL catabolism in vivo.[12] The steroid oxandrolone increased plasma "hepatic" lipase without any appreciable effect on postheparin plasma lipoprotein lipase[13] and, when administered to hyperlipoproteinemic patients (7.5 mg/day for 3 months), levels of VLDL and HDL were reduced, with little effect on LDL.[14] Recent evidence supports the existence of an extravascular hepatic pool of LDL about 20 to 30% the size of the vascular pool.[15] A current theory postulates that the LDL taken up by extrahepatic cells is part of the degradative process and that cholesterol from LDL degradation forms a separate pool which is transported to the liver for excretion.[11]

The role of LDL receptors in the regulation of cholesterol content of normal human fibroblasts has been reviewed.[16] Recent evidence suggests that the major defect in homozygous familial hypercholesterolemia (FH) is not in binding of LDL by cell surface receptors, but in the rate of internalization of LDL, a process resembling absorptive endocytosis.[17] Rate of internalization was 1% to 10% of normal, whereas the rate of surface binding of LDL was reduced by much less. Homozygous FH patients fall into two groups. One responds to diet and drug therapy and the other does not. Fibroblasts from non-responders did not demonstrate LDL-induced suppression of HMG-CoA reductase, whereas the responders did.[18] Leucocytes from patients with heterozygous FH demonstrated a greater induction of HMG-CoA reductase than normals and released more endogenously synthesized cholesterol into the medium.[19]

Relationship of Lipid Metabolism to Atherogenesis - An LDL receptor has been found in cultured cells from the intima and media of the aorta of a human fetus.[20] Since medial cells are believed to be a major site of cholesterol deposition in atherosclerosis, these data raise the possibility that LDL receptors may be involved in cholesterol deposition during formation of the atherosclerotic plaque. Human arterial smooth muscle cells growing in tissue culture, in contrast to rat cells, preferentially bind and take up VLDL and LDL as compared to HDL.[21] These results may be relavent since rat and man differ markedly in their propensity to develop atherosclerosis.

Lipoprotein lipase has been isolated from bovine aorta[22,23] and partially characterized. This enzyme is postulated to act on triglyceride-rich lipoprotein in the arterial wall, forming cholesterol and a cholesterol-ester rich remnant that could be incorporated into the arterial intima. Another postulate is that a high myocardial lipoprotein lipase and low hepatic lipase activity (necessary for the removal of remnant particles) could be the cause of deposition of remnant particles in the aorta.[24]

Epidemiological evidence suggests that the development of atherosclerotic lesions may be accelerated by a decreased clearance of cholesterol from the arterial wall, secondary to a reduction in the plasma concentration of HDL.[25] Serum solubilization of exogenous cholesterol is termed serum cholesterol binding reserve (S.C.B.R.).[26] This binding is believed to be due to two lipoprotein subfractions, one from VLDL and the other from HDL. S.C.B.R. values were recently reported to be lower in patients with premature myocardial infarction than in controls.

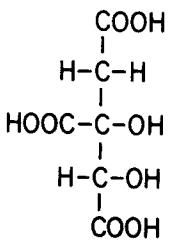
Relationship of Insulin and Glucagon to Hyperlipidemia - It has been postulated that many hyperlipidemic states may be due to an imbalance in insulin and glucagon activity.[27] Cellular resistance to insulin may be central to the development of hypertriglyceridemia.[28] This would lead to a compensatory increase in plasma insulin levels which would stimulate hepatic triglyceride synthesis, thus leading to a rise in plasma triglyceride levels. Among 31 patients with endogenous hypertriglyceridemia, 16 were found to have hepatic steatosis (fatty liver) and a close correlation between plasma insulin levels and liver triglycerides was demonstrated. Insulin administration i.v. showed no change in the decay of plasma insulin, while insulin sensitivity decreased.[29] Clofibrate administration to 13 patients with type IV hyperlipoproteinemia reduced serum triglycerides with a correlated reduction in the insulin/glucagon ratio.[30] Glucagon may function to either hasten the egress of lipoprotein cholesterol from blood to the liver or retard the re-entry of cholesterol from liver into the blood.[31] In rats, glucagon administration has been reported to decrease triglycerides and cholesterol in kidney, aorta and serum.[31,32] Triglyceride and cholesterol content of liver and diaphragm were unaffected.

Mechanism of Action Studies with Lipid Lowering Drugs - Halofenate and clofibrate inhibited the incorporation of radioactive glucose and pyruvate into fatty acids in rat adipocytes and inhibited pyruvate metabolism at or near pyruvate dehydrogenase.[33] Clofibrate inhibited fatty acid and sterol synthesis from acetate in rat and monkey hepatocytes[34] and stimulated the incorporation of both fatty acids and glycerol into lipids.[35] Clofibrate was anti-lipolytic due to inhibition of adenylate cyclase.[36] Clofibrate reversed the elevation of both serum triglycerides and cholesterol but had no effect on the elevated lipolysis in rats with experimental diabetes.[37] The hypocholesterolemic effect of clofibrate was not caused by suppression of hepatic cholesterol synthesis. In rats with experimental nephrotic hyperlipidemia significant hypocholesterolemic effects were demonstrated for halofenate and clofibrate; oxandrolone proved to be significantly hypotriglyceridemic and hypocholesterolemic in combined therapy, due largely to synergistic action with clofibrate-like drugs.[38] Halofenate lowered significantly serum triglycerides and uric acid levels but had no consistent effect on serum cholesterol levels; clofibrate decreased both lipid classes.[39,40] Clofibrate increased the hypocholesterolemic and hypotriglyceridemic effects of cholestyramine and nicotinic acid in patients with type II hyperlipoproteinemia.[41]

A correlation between hypolipidemic activity and peroxisome proliferation by clofibrate and compounds structurally related to clofibrate

(nafenopin, methylclofenapate, SaH 42-348, and S-8527) has been reported. The observation that Wy-14,643 and tibric acid, hypolipidemic agents unrelated structurally to clofibrate, also cause peroxisome proliferation suggests a causal relationship between these two events.[42] However, several studies suggest that peroxisome proliferation induced by clofibrate is independent of its lipid lowering effect. A recent report states that a clofibrate derivative, ethyl α-p-fluorophenoxyisobutyrate is hypolipidemic but does not cause peroxisome proliferation.[43] Nafenopin-[44] and clofibrate-[45] induced peroxisomes showed increases in catalase but decreases or no changes in urate oxidase, L-α-hydroxy acid oxidase and D-amino acid oxidase activities. Peroxisome proliferation by hypolipidemic drugs was correlated with increases in hepatic carnitine acetyltransferase activity.[46] However, a recent study indicated increased activity of this enzyme in only the mitochondrial fraction and not in peroxisomes.[47]

(—)-Hydroxycitrate (1) reduced significantly serum triglycerides in the genetically obese, hyperlipidemic Zucker rat and the fructose-induced hypertriglyceridemic rat.[48] This compound is a potent competitive inhibitor of ATP citrate lyase,[49] a key enzyme involved in the production of acetyl CoA for lipid synthesis, particularly during high carbohydrate intake. (—)-Hydroxycitrate inhibited rates of lipogenesis in vivo in liver, adipose tissue and small intestine in rats.[50,51] Chronic oral administration decreased weight gain through a reduction in body triglycerides; these changes were correlated with a reduced food intake (see Chapter 21).[52]



(—)-threo-hydroxycitric acid

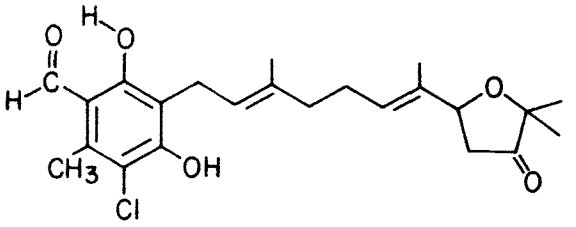
(1)

Neomycin (2 g/day) administered to 4 patients produced a significant 25% reduction of plasma cholesterol accompanied by a significant increase in fecal sterol excretion, consistent with a decreased rate of cholesterol absorption.[53] In a double-blind trial a greater reduction in serum cholesterol compared to placebo, was obtained in severe and mild hypercholesterolemic patients treated with probucol and neomycin.[54] Probucol transiently increased the fecal excretion of bile acids, while neomycin enhanced the fecal elimination of cholesterol as neutral sterols. Recent clinical trials showed colestipol to have useful hypocholesterolemic activity.[55,56]

Ascofuranone (2), a fungal metabolite, reduced plasma cholesterol in rats and lowered hepatic and cardiac cholesterol content without affecting body weight gain in rats fed normal or cholesterol rich diets.[57] Its mode of action appears to be both a stimulation of cholesterol catabolism and an inhibition of exogenous cholesterol absorption.

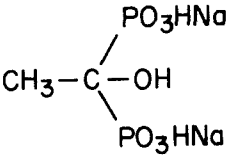
The role of calcification in the pathogenesis of experimental atherosclerosis was investigated using disodium ethane-1-hydroxy-1,1-diphosphonate (EHDP, 3) which influences calcium metabolism.[58] EHDP administered daily

to rats beginning with the initiation of the atherogenic regimen caused a dose-related inhibition of arterial calcification at 8 weeks.[59] No apparent effect on serum cholesterol and triglyceride levels was observed. After 3 months of oral treatment with EHDP (1.2 g/day), patients with various bone diseases demonstrated a significant reduction in plasma cholesterol. Cholesterol and triglycerides decreased in other hyperlipidemic subjects with no bone disease.[60]



ascofuranone

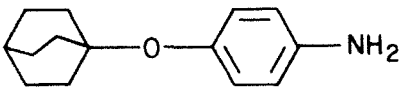
(2)



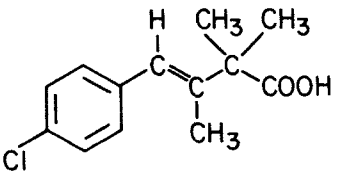
EHDP

(3)

New Agents - A number of new compounds demonstrating hypocholesterolemic activity were described. These include derivatives of eritadenine,[61] analogs of MER-29,[62] and p-(1-bicyclo[2.2.2]octyloxy)aniline (4).[63] The p-chloro analog of α,α-dimethyl-β-benzal-butyric acid (5)[64] was reported recently to inhibit cholesterol synthesis.

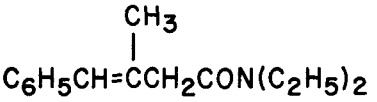


(4)

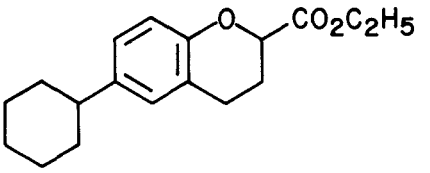


(5)

Several types of compounds were reported to have a specific hypotriglyceridemic effect. These include 3-methyl-4-phenyl-3-butenoic acid diethylamide (6)[65,66] and ethyl-6-cyclohexylchroman-2-carboxylate (7).[67] S-8527 reduced serum and liver triglyceride levels and inhibited the conversion of [^{14}C] acetate into triglyceride and phospholipids, due to a suppression of acetyl CoA carboxylase.[68]

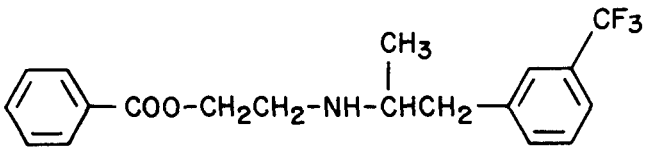


(6)



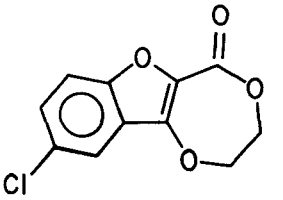
(7)

New compounds reported to reduce both blood cholesterol and triglyceride levels include 780 SE (8), a fenfluramine derivative;[69] DH-990, a probucol analog;[70,71] a benzofuran (9) structurally related to clofibrate;[72] and several N-benzyl-imidazoles (10), the 3-methoxy, 4-methoxy and 4-methylbenzyl derivatives being most active.[73] Certain 1-alkylimidazoles (11) have been shown to inhibit cholesterol synthesis.[74] Hypolipidemic activity of two prostanoic acid derivatives was reported.[75,76]

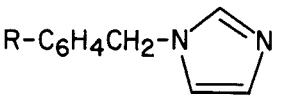


780 SE

(8)

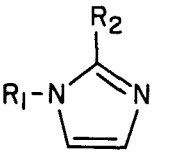


(9)



R
3-OCH_3
4-OCH_3
4-CH_3

(10)



R_1 = geranyl, farnesyl, n-decyl, n-dodecyl, n-tetradecyl

R_2 = H, CH_3

(11)

Cholesterol Gallstones: Pathogenesis - The current concept implicating a hepatic defect in the pathogenesis of gallstones has been comprehensively reviewed.[77,78] Controlled studies have confirmed that an important etiologic factor for cholelithiasis is the hepatic secretion of a bile with an increased ratio of cholesterol to bile acids and lecithin (i.e., supersaturated bile or lithogenic bile). Patients with gallstones have increased

hepatic secretion of cholesterol,[79] decreased bile acid secretion rate[79] and pool size,[80] smaller biliary pools of cholic acid and chenodeoxycholic acid (CDC)[80,81] a reduction in CDC synthesis,[81] and a decline in the catabolism of cholesterol to cholic acid and CDC.[80] The possible involvement of an extrahepatic abnormality in the production of lithogenic bile has also been considered.[82] An association of cholelithiasis with obesity[79,83] and type IV hyperlipoproteinemia[84] has been demonstrated. Clofibrate treatment resulted in increased lithogenicity of bile,[85] and thus may induce gallstone formation.[86-88]

Dissolutione of Gallstones by CDC - The subject of dissolution of gallstones by means of chemical agents, particularly CDC has been recently reviewed.[89] Long-term administration of CDC to patients with radiolucent gallstones and intact gallbladder function, at doses of up to 1.5 g/day, resulted in partial or complete dissolution of gallstones in some.[90,91] A national effort has been initiated to assess the safety and efficacy of CDC in patients with gallstones.[92] The addition of phenobarbital[93] or β-sitosterol[94] may have some advantage over CDC alone on gallstone dissolution. Successfull gallstone dissolution has also been achieved with infusion of sodium cholate,[95] and oral administration of ursodeoxycholate acid.[96]

Metabolic Effects of CDC - A review[97] may be consulted for earlier studies. In patients responding to treatment with CDC, cholesterol saturation was significantly reduced.[91,98-100] Hepatic HMG-CoA reductase and cholesterol 7α-hydroxylase activity declined following CDC therapy.[99] A significant reduction of daily biliary outputs of cholesterol occurred, while bile acids and phospholipids were unchanged.[100] Treatment with CDC expanded the pool size of CDC.[100,101] At least 80% of the total biliary bile acids was composed of CDC,[100,102] chiefly conjugated with glycine.[101] The pool sizes of cholic acid and deoxycholic acid were decreased,[98,100-102] as was cholic acid[101,103] and endogenous CDC[103] synthesis.

Although on the basis of animal studies (see below), lithocholic acid (LC) has been conjectured to play an important role in the potential hepatoxicity of CDC in man, the effect of CDC on LC metabolism in gallstone patients has not been studied rigorously. Sulfated LC has been found to increase considerably.[101] Recent studies[104-106] in healthy subjects suggest that LC is absorbed from the distal intestine and is completely conjugated and partially sulfated before secretion into bile, that sulfated LC is rapidly excreted in the feces, and that LC and its metabolites are rapidly cleared from the plasma. Treatment with CDC gave also rise to increments in ursodeoxycholic acid,[98,102] presumably synthesized from CDC through 7-keto-lithocholic acid as an intermediate.[102]

Hepatotoxicity of CDC - CDC was hepatotoxic in baboons when administered at 20 to 38 mg/kg/day for 8 to 15 months. Liver damage was manifested both enzymatically and histologically.[107] Pregnant Rhesus monkeys given CDC developed diarrhea, vomiting, appetite suppression, and weight loss.[108] Fetuses showed changes characterized by vasodilation of liver, kidney, and adrenals. In addition, there were varying degrees of hepatic cell necrosis,

and renal and adrenal hemorrhage. In Rhesus monkeys given CDC for one month, the only significant change was an increase in serum leucine amino peptidase (SLAP) and biliary LC,[109] but continued treatment for 15 weeks[110] or six months[111,112] resulted in severe liver damage in addition to elevations of SLAP, serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), and biliary LC. Thus an elevation of SLAP activity appeared to be an early manifestation of hepatotoxicity induced by CDC via its metabolite LC. Severe liver damage was also produced in rabbits fed CDC or LC for 3 to 21 days,[113] and pathologic changes correlated with the increases in biliary LC. Transient increases in SGOT levels[90,91,98,114] and both increases[99,101] and decreases[98,101] in LC have been observed following CDC therapy in man. The minor changes observed in some liver biopsy samples could not be attributed to CDC therapy with any degree of certainty.[90,91,114,115]

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Chapter 20. Drug Metabolism

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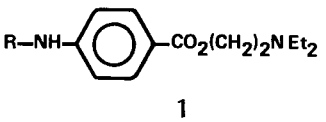
Introduction – This chapter is a departure from reviews on this subject in previous years, which were essentially a compilation of drug biotransformation products. We have attempted to illustrate, by means of examples from the literature of the past two years, how drug metabolism (pharmacokinetic and biotransformation) data may be used in directing the design of new chemical entities. The fact that metabolic events frequently lead to active or toxic metabolites merits consideration by the medicinal chemist as does information on new or little studied biotransformation routes. Species differences in drug metabolism are also addressed, since they bear directly on the relevance of efficacy and toxicity observed in animals to usefulness in man. Drug metabolism studies are of greatest value to the drug discovery process when they are carried out early and permit rapid feedback to the synthetic program. Although many of the studies discussed here were performed following evaluation of the respective agents in man, they nevertheless reveal the uses to which metabolism information can be put in the rational design of drugs. An understanding of general metabolic parameters should also be useful for predicting the metabolic fate of compounds to be synthesized.

Recent reviews which may be of more general interest deal with distribution and metabolism of drugs in the lungs[1], the relationship of drug metabolism and disposition to safety[2,3], drug interactions[4], hepatic esterases and amidases[5], metabolic factors in liver toxicity[6], hemodynamic factors in drug disposition[7], and the blood-brain barrier[8].

Methodology – Advances in methodology have made possible the collection of data at stages in drug development where it can be of greatest value to the discovery effort. Several techniques, gaining in popularity, are worthy of mention. The combination of mass spectrometry with gas chromatography, permitting vastly superior assay sensitivity and specificity, has been reviewed[9]; the power of this approach has been demonstrated in studies with propoxyphene[10] and prostaglandins[11]. Assay of drug and metabolites in saliva, permitting pharmacokinetic determinations where plasma sampling is difficult, are exemplified by studies with tolbutamide[12], heroin [13], digoxin[14], and theophylline[15]. Microbial systems to model mammalian drug metabolism, recently reviewed[16], show considerable promise in the elucidation of biotransformation pathways and in the preparation of quantities of specific metabolites for further study. Isolated organ preparations are now frequently used in the study of biotransformation uncomplicated by distributional and excretory phenomena. Liver[17], lung, kidney, and pancreas have been used[18-20]. Methods for the isolation and use of hepatocyte preparations have been refined[21,22] and should find increased use. Human fetal and placental tissues have been employed to study the metabolism of compounds which could not otherwise be studied in man[23,24].

Relationships between Structure and Pharmacokinetics – Potency differences in members of a chemically-related series of compounds depend not only on intrinsic potency at the receptor site but also on the factors which influence the ability of the drug to reach and remain at the receptor. These factors include rate and extent of delivery of drug to the site of action and rate of removal therefrom, as determined by the pharmacokinetic and biotransformation properties of the drug. Therefore, rational drug design must take into account these metabolic influences. Correlations of hydrophobic, electronic, and steric parameters with intrinsic potency will be useful and meaningful only when extended to drug kinetics and biotransformation. Some recent reviews of general interest in this respect have appeared. Data on 186 drugs were tabulated with regard to plasma half life and urinary recovery, including changes resulting from decreased renal and hepatic function[25]. The relationship of structure to various pharmacokinetic parameters has been discussed[26,27] and the use of prodrugs to modify oral absorption reviewed[28].

The influence of structure on oral absorption, tissue distribution, and protein binding has been examined in several series of compounds. The rate of intestinal absorption of a homologous series of carbamates was found to correlate with the partition coefficient, peaking at n-butyl[29]; with N-methyl carbamates this relationship no longer held, possibly due to a change in hydrogen bonding characteristics[30]. However, absorption rates from the stomach could be directly related to the partition coefficient of both series of homologs, suggesting a simpler absorption process in that organ. The relative protein binding of a series of local anesthetics was highly dependent on the length of the side chain[31], peaking at C-3 for benzoic acid derivatives (**1**) and at C-4 for cinchocaine analogs (**2**). Binding to bovine, rat, and human serum albumin of several cyclic and acyclic analogs of the hypolipemic drug clofibrate could be correlated with drug transport but not with hypolipemic activity[32]. From studies with a large series of disopyramide analogs, structural variations on all four functional groups around the tetrahedral carbon were examined, yielding linear equations relating human plasma binding constants with lipophilicity[33]. Differences in regional distribution of saturable binding sites in rat brain have been described for dihydromorphine vs. naloxone and related to function of the opiate receptor[34]. Using trans- and cis-dimethylaminostilbene, dimethylaminodibenzyl, and 3-methylcholanthrene, it was shown that certain structural features can sometimes override the influence of lipid solubility on absorption via the intestinal lymphatic system of rats[35]. The greater embryotoxicity of diquat as compared to paraquat was correlated with enhanced distribution of the former into the mouse fetus[36]. Distribution studies with three chemically-related β-adrenergic blocking drugs in the brain showed propranolol to be concentrated in the hippocampus and pindolol in the septum while sotalol was not significantly concentrated centrally[37]. Since all three drugs have similar pharmacological activity, these findings may have significance in relation to a possible central contribution to the mode of action of these drugs. The permeability of various substances across the blood-testis barrier of the rat was found to be related to molecular size for nonelectrolytes and to pKa for acidic drugs[38].



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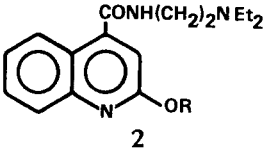

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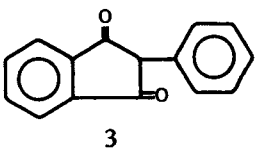
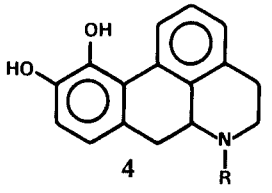


3

The half life of phenindione (**3**) in man was shorter than that of the p-fluoro analog, perhaps due to greater susceptibility of the former to biotransformation[39]. Appreciable differences in half lives were also noted for the p-methoxy, p-bromo, and p-chloro analogs of **3**[40]. Distribution volumes were similar except for the methoxy analog which was more highly bound to plasma proteins. The influence of secondary alcohol vs. ketone, para-alkylation, and branched vs. straight chain alkyl groups on distribution was examined in a series of derivatives of mandelic acid and benzoylformic acid[41]; differences in lipophilicity were shown to account for some of the changes observed. Distribution into mouse brain increased with increasing chain length in norapomorphine (**4**, R = H), apomorphine (**4**, R = CH_3), and N-n-propylnorapomorphine (**4**, R = C_3H_7) but plasma half lives did not follow this order[42]. In the rat the S isomer of warfarin has a longer plasma half life than the R isomer[43]; in man the R isomer has the longer half life[44].



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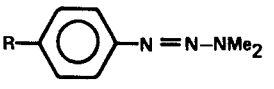
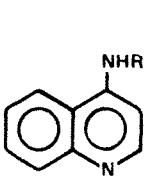


5

Relationships between Structure and Biotransformation — Several studies have addressed the influence of homology and substituents on the rate and route of biotransformation. In an *in vitro* study examining the

effect of ring size on hydrolysis, adipimide was found to be a much better substrate for enzymatic cleavage than succinimide or glutarimide[45]. Substitution is a factor, however, as α-phenylsuccinimide (the N-demethyl metabolite of phensuximide) is rapidly hydrolyzed. In a series of N-alkylamphetamines and N,N-dialkylamphetamines the rate of dealkylation in man was shown to increase with chain length[46]. In the former series the rate varied with the logarithm of the number of carbons in the alkyl chain (except for the 2-propyl and 2-butyl compounds where steric factors interfered) while in the latter it was related to the square of lipid solubility. Steric hindrance due to increased branching in the acyl moiety was found to be a determining factor for the rate of hydrolysis of several terbutaline esters[47]. The oxidative demethylation by rat liver microsomes and supernatant fractions of some p-substituted phenyl triazines (5) with antineoplastic activity has been shown[48] to proceed in the order CH_3 >OCH_3 >$CONH_2$ >H >COOEt >COOH. In a comparison of the antimalarial compounds chloroquine (6) and amodiaquin (7), N-desethylation of amodiaquine by rats was found to proceed much more rapidly than that of chloroquine[49]. The dramatic influence of neighboring groups on metabolism was demonstrated in humans with nealbarbitone (8, R = $CH_2C(CH_3)_3$), the allyl group of which is extensively oxidized to the diol[50]. In contrast the structurally related quinalbarbitone (8, R = $C(CH_3)H(CH_2)_2CH_3$) undergoes 3-hydroxylation of the methylbutyl side chain with loss of the allyl group.



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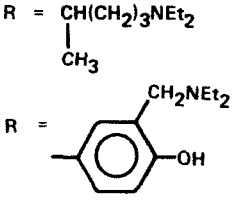

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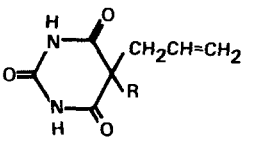


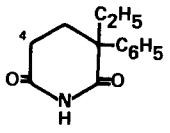
8

N-Demethylation of tripelennamine is largely blocked in rats by N-nitrosation although aromatic hydroxylation continues[51]. In an examination of the influence of substitution on the susceptibility of drugs to microfloral degradation[52], phenacetin and closely related analogs (but not other N-acylated compounds) were deacylated; no N-dealkylation was seen. Studies with several alkyl and alkoxy pyridines and pyrazines in rats demonstrated that, although alkyl groups were oxidized to carboxylic acids in both series, conjugation with glycine was more extensive in the pyridine series[53]. Compounds with adjacent alkyl groups were ring hydroxylated while methoxy analogs were demethylated in both series. The relative ease of hydrolysis of twelve carbenicillin esters was examined *in vitro* and related to human and monkey serum concentrations of carbenicillin following oral administration[54]. Aryl esters were appreciably more labile than alkyl esters. In a number of hexapeptide analogs corresponding to residues 4 to 9 of ACTH differences in behavioral potency could be correlated with rates of biotransformation[55].

A number of studies have dealt with isomer effects on biotransformation. The position of ring substitution can influence metabolism as revealed by the fact that 2-naphthol, but not 1-naphthol, is hydroxylated by the cat[56]. In the pig 1-naphthol yields both sulfate and glucuronide in similar quantities while the 2-isomer gives almost exclusively the glucuronide. Ifosfamide, which differs from cyclophosphamide by transposition of a chloroethyl group from the exocyclic to endocyclic nitrogen, is metabolized by man at a markedly slower rate than cyclophosphamide, possibly accounting for its greater antineoplastic activity[57]. Although both R- and S-warfarin are reduced in man to alcohols and oxidized at position 6, only the S isomer is oxidized at the 7 position[58]. Using human and rat cytosols from liver and kidney, the reduction of the R isomer was shown to proceed at a faster rate than that of the S isomer[59]. R-warfarin was reduced mainly to the RS isomer, whereas S warfarin yielded the two diastereomeric alcohols in approximately equal amounts. Metabolism studies with R and S-N-isopropylamphetamine by rat liver microsomes suggest that oxidation at the α-position is the rate-limiting step in dealkylation and deamination of secondary amphetamines of the S configuration, whereas metabolism of the R isomers is based on N-oxidation[60]. Stereochemistry is unimportant for N-dealkylation of tertiary amphetamines. Following administration of a racemic mixture of

the stereoisomers of ibuprofen to man, similar peak plasma concentrations of each isomer were seen but concentrations of the l-isomer declined more rapidly[61]. Since urines of all patients contained more of the d-isomer, this suggests more rapid metabolism of the l-isomer. In an examination[62] of the four stereoisomers of 3,4-dihydroxyphenylserine, rates of formation of norepinephrine by rats demonstrated the αS configuration, regardless of configuration of the β position, led to more rapid decarboxylation.

Metabolic Activation – Literature continues to provide examples of metabolites that contribute extensively to the pharmacological activity of parent drug. Several drugs are activated or maintain their pharmacological effects on hydroxylation. The relationship between metabolic 3-hydroxylation and anticonvulsant activity of the 1,4-benzodiazepines has been extended to bromazepam[63], the 3-hydroxy metabolite of which has comparable intrinsic activity, although its uptake by the brain of mice appears to be less efficient. A 4-hydroxylated metabolite of glutethimide (9) in man[64] possesses twice the sedative-hypnotic activity of parent drug in mice. Extensive research continues to explore whether the 11-hydroxy metabolite can solely account for the pharmacological activity of Δ^9-tetrahydrocannabinol (THC)[65]. These studies have revealed that 11-hydroxylation is not a prerequisite for general cannabinoid activity[66] but may be essential for the analgesic component of THC[67]. Recent reviews have summarized the wealth of current knowledge on the disposition of Δ^9-THC in animals[68,69] and man[69,70].



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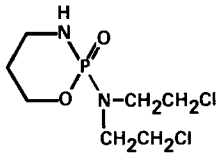

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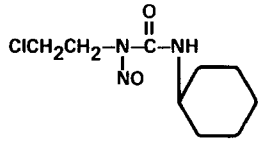
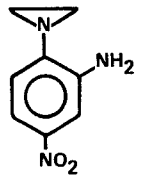


11

Efforts to clarify metabolic events concerned with activation of cyclophosphamide (10) and analogous antitumor and immunosuppressant agents of the 1,3,2-oxazaphorinane class continue[71]. Initial hydroxylation, postulated to occur in the C-4 position of cyclophosphamide was confirmed in studies using rat liver microsomes[72]. Hydroxylation of the cyclohexyl ring may be primarily responsible for the anticancer activity of the substituted nitrosourea CCNU (11)[73]. The antitumor activity of the dinitrophenyl aziridine CB 1837 may be dependent on hepatic reduction to compound **12** [74]. The catechol metabolite of piribedil (13) in rats and man is thought to be responsible for the dopaminergic agonist activity of this agent, although metabolite concentrations in rat striatum following piribedil administration were reported[75] to be considerably lower than those activating adenylcyclase *in vitro*. Both 6-β-hydroxynaltrexone, a major urinary metabolite[76], and 2-hydroxy-3-methoxy-6-β-hydroxynaltrexone, a less prevalent but more lipophilic metabolite[77], have been implicated in the prolonged action of the narcotic antagonist naltrexone (14) in man.



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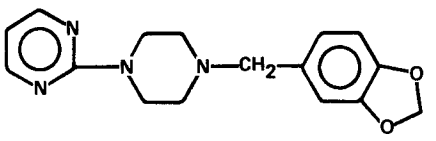

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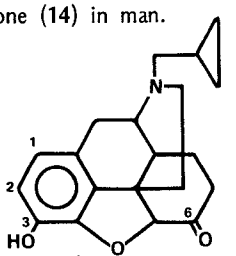
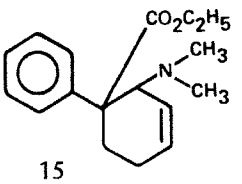


14

A close relationship between N-monodemethylation and the analgesic effects of tilidine (15) in the rat has been observed[78]. Acetylmethadol (16) is N-di-demethylated in man to a primary amine, which, like the N-monodemethylated metabolite, exhibits potent analgesic activity in mice[79], and may contribute extensively to the prolonged opiate effect of this drug[80].



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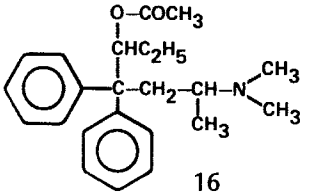
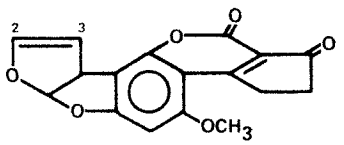


16

The prodrug concept, recently reviewed [28], continues to provide new approaches to drug delivery. A more recent example is the dihydropyridine derivative of the hydrophilic quaternary salt N-methylpyridinium-2-carbaldoxime chloride (2-PAM), which facilitates passage of the blood-brain barrier and is rapidly oxidized to the active form[81].

The frequency with which the toxicity of drugs and other chemicals is mediated by metabolism and covalent binding of a reactive species to tissue macromolecules[82] has stimulated considerable study of the biotransformation of a number of new and important agents. Increasing evidence suggests that intermediate epoxide formation is an important metabolic transformation of xenobiotics that contain an aromatic or olefinic double bond. The ultimate carcinogen of aflatoxin B_1 (**17**) would appear to be an epoxide formed at the 2,3-double bond which binds to RNA; a related, less carcinogenic aflatoxin in which the 2,3-double bond is saturated does not bind to macromolecules[83]. The allylic double bond of the flavoring agent safrole (**18**)[84] and the double bond of diethylstilbestrol[85] undergo metabolic epoxidation based on animal urinary metabolite analyses. Concern about hepatic side effects of the contraceptive agent norethindrone, coupled with knowledge that this agent forms a 4,5-epoxide metabolite, led to investigations concluding that activated drug metabolites were irreversibly bound to tissue protein[86].



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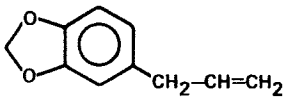

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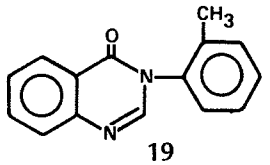
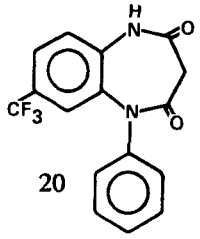


19

The phenyl ring of glutethimide (**9**)[87] and the tolyl moiety of methaqualone (**19**)[88] are extensively metabolized in man by the epoxide-dihydrodiol pathway. The 5-phenyl substituent of the 1,5-benzodiazepine triflubazam (**20**), unlike that of the 1,4-benzodiazepines, is extensively hydroxylated in several species with evidence of arene oxide mediation[89]. As with drugs containing phenyl groups, epoxidation of the naphthalene moiety can be an important metabolic event, as was shown in animal studies with the β-adrenergic blocking agent pronethalol[90]. Urinary metabolites of antipyrine (**21**) identified in animals and man[91] indicate that the double bond of the pyrazolinone ring, but not the aromatic ring, is extensively hydroxylated by the epoxide-diol pathway. Evidence for the 10,11-epoxidation of tricyclic agents has been extended to protriptyline (**22**)[92] and cyproheptadine (**23**)[93]. The identification of appreciable quantities of intact 10,11-epoxides in the urine of rats suggests that the formation of these metabolites may represent a true detoxification mechanism[94]. A new pathway for arene oxides has been described involving reduction back to parent hydrocarbon with an epoxide reductase found in rat liver microsomes[95]. The role of this enzyme in modifying the carcinogenicity of certain aromatic compounds needs to be explored.



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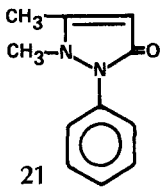

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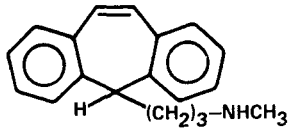

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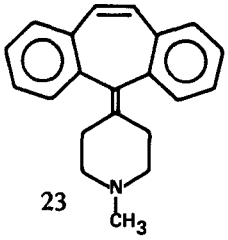

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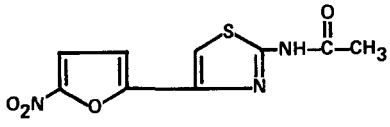

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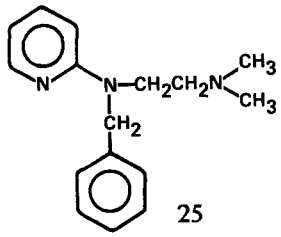
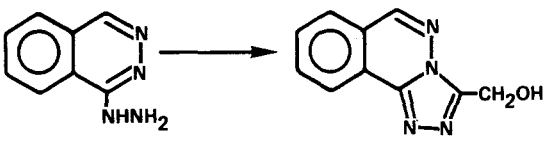


25

A better understanding of the metabolism of drugs containing aromatic nitro groups is of continuing interest in view of the severe toxicity these compounds can often cause. This is exemplified with several carcinogenic antimicrobial 5-nitrofurans and 5-nitrothiophenes, including NFTA (**24**) which upon nitroreduction firmly binds to rat microsomal protein[96]. The toxicological significance of drug-nitrite interactions to form nitrosamines remains to be clarified, as does the mechanism of metabolic activation[97]. N-Nitroso metabolites of drugs containing secondary and tertiary amine groups may not necessarily mediate tissue lesions, as recent studies with tripelennamine (**25**) suggest[51,98]. A major metabolite (**27**) of hydralazine (**26**) has been identified[99] which, through irreversible protein binding, may help to explain incidences of lupus erythematosus induced by this drug.



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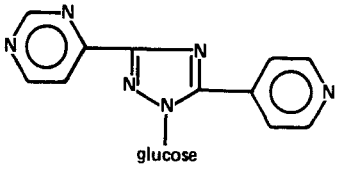
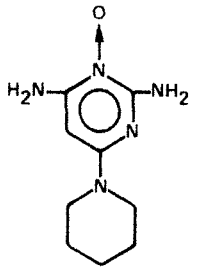


28

New Pathways of Metabolism – A number of publications which contribute knowledge about novel or little studied pathways of drug metabolism merit attention. The glucosylation and glucuronylation of heterocyclic nitrogen has been established as a mammalian pathway of metabolism. A β-glucoside (**28**) of a 3,5-disubstituted triazole[100] with prolonged xanthine oxidase inhibitory activity, and an apparent quaternary ammonium glucuronide of cyproheptadine (**23**)[101] are major human metabolites. A glucuronide is the major excretory product of the hypotensive agent minoxidil (**29**) in monkeys and man[102]; linkage likely occurs through the hydroxy group of the N-hydroxy tautomer of the pyrimidine ring N-oxide. The first reported examples of C-β-glucuronides derived metabolically from drugs have been those of sulfinpyrazone and phenylbutazone, where linkage occurs on the C-4 position of the pyrazolidine ring[103]; this is not surprising in view of the acidic character of the enolizable C-4 proton.



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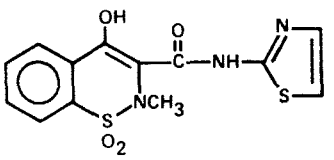

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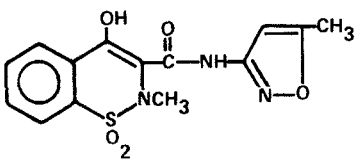
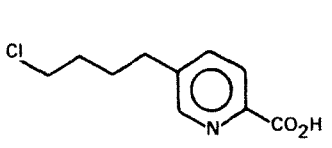


31

The major metabolites of the anti-inflammatory agent sudoxicam (**30**) in rats, rabbits and monkeys arise from scission of the thiazole ring[104], while isoxicam (**31**) is metabolized (dogs, rats) extensively by benzothiazine cleavage to a glyoxylamide[105]. The S-oxidation of compounds containing thiourea moieties to sulfur-free substances may represent a new metabolic pathway[106], reminiscent of that of the amine oxidases.

A substituted picolinic acid (**32**) with hypotensive activity reportedly undergoes major biotransformation in several species including man by an unusual 2-carbon chain elongation process[107], similar to that of endogenous fatty acids, at the carboxyl group to yield the metabolites -$(CH)_2CO_2H$, -$CH{=}CHCO_2H$ and -$COCH_2CO_2H$. Epimerization at saturated carbon by an R-aryl-propionic acid isomerase has been postulated in man[108] for R(-)-p-*iso*-butyl hydratropic acid (ibuprofen is the d,l racemate). A novel bicyclic metabolite (**34**) of the antiviral agent acluracil (**33**) was reported as the major urinary product in rabbits and apparently is formed by epoxidation of the double bond, followed by hydrolysis and ring closure[109].



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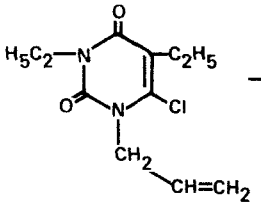

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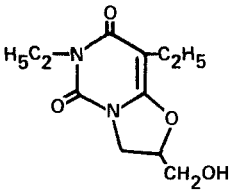


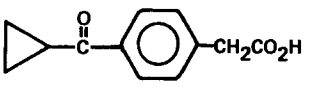
34

The principal pathway of metabolism of the decarboxylase inhibitor carbidopa was established in animals to be loss of hydrazine, probably as N_2[110]. Loss of hydrazine represents a minor pathway of biotransformation in man of the antihypertensive drug hydralazine (**26**)[99,111]. The oxime function, which can be a major intermediate in the oxidative deamination of primary aliphatic amines to ketones, is susceptible to anaerobic reduction to the corresponding hydroxylamine and amine, based on recent *in vitro* studies with acetophenone oxime[112]. Recent literature concerned with the N-hydroxylation of drugs containing primary and secondary amine groups, draws increased attention to the potential pharmacological and toxicological significance of the aliphatic hydroxylamines[113-115].

Comparative Metabolism – The recognition that metabolism may vary with the species employed is important for the extrapolation of animal data to man (see Chapter 25). The problems and considerations involved in the selection of appropriate species for toxicology and drug metabolism studies have been addressed in recent reviews[2,116]. The Rhesus monkey is often considered a suitable model for man[102,117] and increasing interest centers on the squirrel monkey as an alternative to this species[118,119].

Comparative metabolism studies with the tricyclic drugs protriptyline[92] and cyproheptadine[93,120] provide evidence that the rat may have lower levels of epoxide hydrase activity than other animal species. Epoxides are predominant urinary metabolites in the rat, while the corresponding dihydrodiols are excreted by dog and cat. Furthermore, there is no evidence that these epoxides are formed in man[93,101], where glucuronide conjugation is a prominent pathway of metabolism. In contrast, drug metabolism studies with methaqualone suggest that epoxidation in the tolyl moiety represents a major pathway in man but a minor one in the rat[88]. Thus, epoxide formation may depend on substrate specificity as well as the species employed.

The N-hydroxylation of aliphatic amines is also species dependent[121]. Chlorphentermine undergoes extensive N-hydroxylation in the guinea pig, rabbit, rhesus monkey and man, but not in the rat or marmoset[122]. Species differences also occur with respect to reduction of the carbonyl group. Thus, naltrexone (**14**) is reduced substantially to 6-β-hydroxynaltrexol by monkey, guinea pig and rabbit as well as man, but only to a minor extent by mouse, rat and dog[123]. On the other hand, the carbonyl group of the anti-inflammatory agent SQ 20,650 (**35**), is reduced to a much greater extent in the dog than in the rat or monkey[124]. The β-adrenergic blocking agent timolol (**36**) is less extensively metabolized in man (principally



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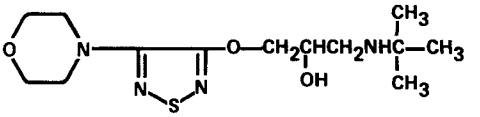

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by morpholine ring cleavage) than by the dog which largely deaminates the drug to a lactic acid derivative[125]. Finally, there is evidence that apparently deficient pathways such as glucuronide formation in the cat and sulfate conjugation in the pig may become activated, as certain phenols are appreciably metabolized in each species[56].

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Chapter 21. Agents for the Treatment of Obesity

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Introduction - Obesity continues to be a major health hazard and the relative long-term ineffectiveness of current therapeutic agents is reflected in the extreme measures being currently employed for treatment of severe cases, e.g. jejunoileal bypass surgery, prolonged starvation, dental splinting. Antiobesity therapy can be achieved either by decreasing the availability of exogenous energy substrates, thereby accelerating the utilization of endogenous ones, or by metabolic regulation, defined in the present context as intervention in the biosynthesis, storage or utilization of energy substrates. In the overall management of the obese patient, anorectic drugs continue to be useful as short-term adjunctive therapy and recent developments in this area will be reviewed. In addition, potential antiobesity agents possessing various metabolic effects which may or may not influence appetite will be discussed.

Current View of the Regulation of Food Intake - Most theories of neural regulation of food intake assume a central excitatory system in the lateral hypothalamus ("feeding center") and a central inhibitory system in the ventromedial hypothalamus ("satiety center"). This model is based on experiments which observed the effects of stimulation and lesioning of these brain areas on feeding behavior.[1a] It is becoming increasingly apparent that such lesions may influence ingestive behavior by interrupting fibers of passage which may or may not synapse on local neurons (for recent review see 2). Pharmacological studies suggest that catecholamines in the brain serve an important neurotransmitter function in the regulation of appetite.[1b]

Advances have been made in the identification of peripheral factors that influence hunger and satiety. The possible importance of hepatic and duodenal glucoreceptors and the mechanism(s) by which signals from these receptors reach the brain, where they are integrated into specific feeding responses, are being examined currently.[3,1c-1e] Several studies suggest that cholecystokinin (CCK) may be involved in the control of appetite,[4,5] possibly by modulating neuronal activity in the hypothalamus.[6] Pharmacological levels of CCK or its synthetic C-terminal octapeptide (SQ 19844) reduced food intake but not water consumption in rats (see Chapter 17 for structure of CCK).[4]

Metabolic Abnormalities in Obesity - A careful assessment of the metabolic, morphological and endocrine differences between obese and lean individuals is important, and could provide new information for the development of

antiobesity agents. The following summarizes the current views of metabolic and endocrine aberrations which characterize this disease and have been reviewed recently.[7a,7b,8,9]

Cellular profiles of the adipose depot of the juvenile-onset obese indicate hyperplasia and hypertrophy compared to lean individuals. Adipose cell profiles in maturity-onset obese suggest predominantly hypertrophy. Rates of triglyceride and cholesterol synthesis and lipolysis are increased in fed obese subjects and these alterations are reflected by enhanced blood levels of triglycerides, cholesterol and free fatty acids. Lipid mobilization and ketosis are decreased in the obese during fasting. Abnormalities in carbohydrate metabolism in obese subjects include hyperglycemia, decreased glucose tolerance, enhanced pancreatic insulin secretion, elevated plasma insulin levels, and increased peripheral insulin resistance. Responsiveness to stimuli which elicit glucagon secretion is reduced in the obese, liver glycogen levels are increased and rates of gluconeogenesis appear to be enhanced. Growth hormone secretory responsiveness is significantly depressed. Cortisol secretion and turnover rates are reportedly elevated. In contrast to lean individuals, obese subjects demonstrate little or no rise in oxygen consumption after exposure to the cold; thermogenesis after a meal is also reduced.

Studies in normal volunteers made moderately obese by forced hyperphagia have demonstrated that many of these metabolic aberrations are at least partially a consequence of corpulence, since they can be reversed by weight loss and induced by overeating.[10] However, there are two important differences between subjects who accumulate fat late in life or by experimentally induced hyperphagia and those with spontaneous hyperplastic obesity. First, the body weight "set point" in these two groups may be different, since normal subjects who gain weight by forced overeating return to their initial lean body weight. Obese individuals encounter great difficulty in maintaining a lower body weight after weight reduction, and in most cases they return to their original obese weight. Second, the hypercellularity of adipose tissue in the obese group cannot be reversed by weight reduction.[9a]

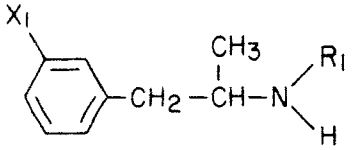
Anorectic Agents: Phenethylamine Derivatives - A comprehensive review[11] has appeared on the pharmacology and therapeutic aspects of fenfluramine (1). Specific reviews should be referred to for more detailed discussions of pharmacology,[9b] mechanism of action,[9b,12a] peripheral and metabolic effects,[12b] CNS effects,[12c] and metabolism.[12d]

The clinical efficacy of fenfluramine in reducing appetite and body weight in obese subjects has been established in several double-blind studies.[13-15,12e,12f] Side effects of fenfluramine are generally mild. Lowered blood pressure and heart rate,[16] mydriasis[16] (probably mediated by the metabolite norfenfluramine),[17] mood depression following end of treatment,[12g] olfactory and visual hallucinations following high doses,[16,18] and fenfluramine abuse[12h,19] have been reported.

Studies on the appetite suppressant activity of fenfluramine and norfenfluramine[20,21] in animals have appeared, and research concerning the

mechanism of action of anorexia predominated during the past year. Whereas the site of action of amphetamine appears to reside in the lateral hypothalamus[22] and to be mediated by adrenergic mechanisms,[23] an extrahypothalamic zone and serotonin (5-HT) mediation appear to be involved in the anorectic action of fenfluramine.[12i,24,25] Several lines of evidence suggest that the anorectic activity of fenfluramine depends upon the release of 5-HT from central neurons. These include: a) decreased brain 5-HT levels following fenfluramine administration;[12j,26] b) drugs which modify brain 5-HT metabolism antagonize the anorectic action of fenfluramine, e.g. chlorimipramine,[27-29] methergoline,[23,27] cyproheptadine,[23] and cinanserin;[23] c) lesions of 5-HT neurons antagonize fenfluramine-mediated appetite suppression, e.g. intraventricular injections of 5,6-dihydroxytryptamine[30] and electrolytic lesions of the midbrain raphe.[31a] Evidence supporting a 5-HT mechanism of fenfluramine anorexia has been demonstrated in man.[12k,32] In other studies, however, p-chlorophenylalanine,[29] methysergide,[27] or destruction of serotonergic neurons[33] all of which deplete 5-HT stores, did not antagonize the effect of fenfluramine on food intake.

The new fenfluramine analog, 780 SE (2), is a potent anorectic agent with mild behavioral effects in rats.[12l] The drug reduced food intake and body weight in obese and non-obese subjects[12m] and significantly improved glucose tolerance in maturity-onset diabetics.[12n] SL 72340 (flutiorex, 3), is an analog more potent than fenfluramine in suppressing food consumption in rats and possessing less sympathomimetic activity. Clinical trials have confirmed its anorectic effects.[31b] Structure-activity studies of a series of phenoxyacetamide derivatives of m-trifluromethylphenylisopropylamine showed that anorectic activity is associated with amine substitution in the para position of the phenoxy moiety. The p-amido analogs, although less potent than the p-amino analogs were devoid of stimulating effects. The most promising compound was the p-acetamido derivative (flucetorex 4).[31c] A number of fenfluramine glycinates (5), although less potent than the parent compound in terms of anorexia, were less toxic and almost devoid of



	X_1	R_1	
fenfluramine	CF_3	C_2H_5	(1)
780 SE	CF_3	$(CH_2)_2OCC_6H_5$ (O)	(2)
SL72340	SCF_3	C_2H_5	(3)
flucetorex	CF_3	$CCH_2OC_6H_4NHCCH_3$ (O, O)	(4)
fenfluramine glycinate	CF_3	CH_2COOH	(5)

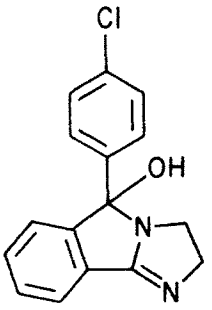
sedative properties.[9c]

Double-blind clinical studies with diethylpropion have established that a single daily dose of 75 mg produced weight loss without any adverse effects on electrocardiogram, chest X-ray, blood pressure, or pulse rate.[34-37,9d] Diethylpropion was rapidly and extensively metabolized by N-de-ethylation and stereoselective carbonyl reduction.[38,39]

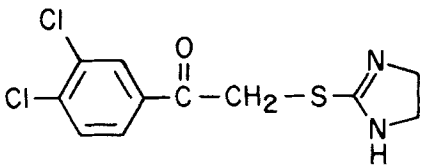
Phentermine produced significantly greater weight loss than placebo in a double-blind study.[40] Chlorphentermine also caused significant anorexia in man.[41] Both phentermine and chlorphentermine are metabolized via N-oxidation to the corresponding hydroxylamino, nitroso and nitro compounds;[42] however, there were marked differences in tissue accumulation. Unlike phentermine, chlorphentermine accumulated in tissues which possess a high content and/or rapid turnover of phospholipids, e.g. CNS, retina, lungs, testes[43-48] and was associated with phospholipidosis in these tissues. A complex formation between chlorphentermine and phosphatidylcholine has been reported; no such interaction was observed with phentermine.[49] This complex formation could prevent normal breakdown of phospholipids by phospholipases, thus leading to an increased intracellular accumulation of lipid, i.e. phospholipidosis.

Anorectic Agents: Non-Phenethylamine Derivatives - Double-blind studies with mazindol (6) demonstrated effective weight reduction in obese patients.[50-55,9e,9f] Mazindol (3 mg/day) was equal to phenmetrazine (75 mg/day),[56] equal to[56] or superior to[57] d-amphetamine (15 mg/day), superior to diethylpropion (75 mg/day)[58] and equal to fenfluramine (up to 160 mg/day)[59] in producing weight loss. CNS stimulation has been shown with mazindol in rats, cats and monkeys, but at doses higher than those evoking appetite suppression.[60] Other pharmacological actions included minor effects on blood pressure and heart rate of rats and dogs, potentiation of norepinephrine pressor responses, antagonism of reserpine-induced hypothermia in mice and tetrabenazine-induced catalepsy in rats, and suppression of muricidal behavior in rats.[60] These and other pharmacological responses suggest that mazindol produces alterations in brain norepinephrine metabolism, primarily through inhibition of the neuronal uptake mechanism, thus increasing norepinephrine availability for receptor neurons.[61] Unlike amphetamine, mazindol does not release norepinephrine from neuronal stores, nor inhibit norepinephrine synthesis. Other evidence suggests that mazindol exerts its anorectic effect by directly stimulating the central dopamine receptors[31d] possibly by enhancing dopamine availability at receptor sites.[9b] Structural modifications of mazindol resulted in a loss or decrease in anorectic activity.[62]

DITA (7) was reported to reduce appetite and increase spontaneous motor activity in rodents.[63] Its potential for abuse was evaluated in Rhesus monkeys where it was found to be 1/3 as potent as d-amphetamine.[64]

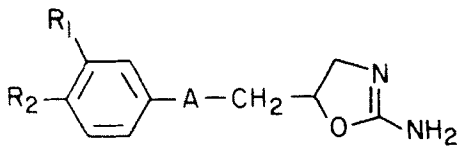


mazindol (6)



DITA (7)

APMO (8), although structurally unrelated to amphetamine, has a similar pharmacological profile, and is less potent than d-amphetamine; both compounds cause anorexia in several species, increase spontaneous motor activity in mice and increase blood pressure in anesthetized dogs.[65] On a mg/kg basis, APMO produced less pronounced pressor responses on arterial and pulmonary blood pressure than amphetamine[66] or aminorex.[67] The anorectic activity in rats of several analogs has been described.[68] Compound 9 was the most active, although less potent than d-amphetamine.



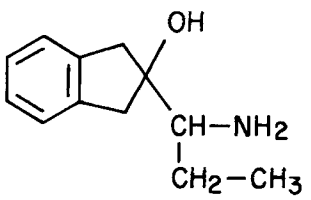
	R_1	R_2	A	
APMO	Cl	Cl	O	(8)
	H	H	S	(9)

11698 JL (10), a novel aminopropylindanol analog, reduced body weight in obese patients on a restricted diet when given at a dose of 120 mg t.i.d. in a double-blind study.[9g]

A recent study demonstrated that l-dopa (200 mg/kg) significantly reduced food intake in rats, and this effect could be increased markedly by employing simultaneously a decarboxylase inhibitor.[69]

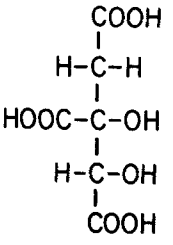
Metabolic Effectors - (—)-Hydroxycitrate reduced food intake, weight gain and body lipid levels in lean rats, obese rats, and mice.[70,71] This compound was initially reported as a potent competitive inhibitor of ATP citrate lyase, the extramitochondrial enzyme which cleaves citrate to acetyl CoA and oxaloacetate.[72] As expected from this activity, subsequent studies demonstrated marked reduction of fatty acid and cholesterol synthesis by (—)-hydroxycitrate in rats.[73,74] The reduction in food consumption produced by (—)-hydroxycitrate occurred concomitantly with an alteration in metabolic flux of dietary nutrients. Carbohydrates and their metabolites were diverted from lipid synthesis,[1f,73,74] and simultaneously significant increases in hepatic glycogen synthesis and levels were observed.[1f] (—)-Hydroxycitrate suppresses appetite possibly by influencing the

activity of hepatic glucoreceptors.[1f]



11698 J L

(10)



(−)-threo-hydroxycitric acid

(11)

Recent studies suggest that the inhibition of carbohydrate digestion in the intestinal tract by inhibitors of pancreatic α-amylase may provide a possible approach to the treatment of obesity and diabetes mellitus. An inhibitor from wheat, Bay d 7791,[75] consisting of thermostable proteins with a molecular weight of about 16,000, and one from microorganisms, Bay e 4609,[76] comprised of complex oligosaccharides, have both been found to diminish the intestinal digestion of starch,[77,9h] and to attenuate the hyperglycemic and serum insulin response to starch ingestion in healthy volunteers,[75,76,9h] and diabetic patients.[75] The conversion of starch carbohydrates into lipids of adipose and aortic tissues of rats was reduced by the oral administration of Bay e 4609.[9i]

Fenfluramine and mazindol in addition to their anorectic activity possess effects on energy metabolism, which although of limited significance in explaining their antiobesity action, may suggest other mechanisms by which drugs could control obesity. Fenfluramine has been shown to interfere with lipid metabolism at several points: 1) a reduction of glyceride synthesis has been observed in rat liver[9j] and in human adipose tissue[78] (K_i ∿1 mM) and was attributed to inhibition of phosphatidate phosphohydrolase;[9j,79] 2) inhibition of the intestinal absorption of dietary triglycerides was reported,[80] which was believed to be due to inhibition of intestinal palmitoyl CoA:monoolein acyltransferase (K_i ∿2 mM);[81] fenfluramine also reduced pancreatic lipase (K_i <7 mM)[82] and stimulated this enzyme at ≤1 mM;[83] 3) fenfluramine and derivatives (100 mg/day for 3 days) diminished significantly triglyceride secretion from rabbit liver.[12o] These effects, however, occurred at a K_i (∿1 mM) 10,000 times greater than the concentration of fenfluramine in plasma at therapeutic doses.[84] Furthermore, a recent study in man demonstrated that neither fenfluramine (40 to 120 mg/day) nor 780 SE (240 to 720 mg/kg) significantly altered fat excretion.[12p]

Fenfluramine significantly increased insulin-dependent glucose uptake by isolated human skeletal muscle[9k,85] and rat hemidiaphragm[9k,12q] without affecting glycogen content at concentrations similar to blood levels (∿100 ng/ml) in patients on a therapeutic regimen of fenfluramine. Similar results have been shown for flutiorex.[86] Fenfluramine was also shown to mimic the effect of insulin on glucose oxidation by human adipose tissue.[12r]

No major effect of fenfluramine on blood glucose or insulin levels was evident in human subjects on restricted diets.[12s,12t]

Recent studies demonstrated that acute treatment with mazindol (4 mg) produced significant improvement in oral glucose tolerance and a concomitant reduction of insulin secretion, without an effect on intravenous glucose tolerance[87,97] which suggests that mazindol interfered with intestinal glucose absorption.

Although hormonal therapy of obesity is controversial,[88] the use of these agents may increase the understanding of hormonal regulation in obesity. As mentioned previously, growth hormone levels are reduced in obese subjects. However, although l-dopa is known to stimulate growth hormone release in normal man, the administration of l-dopa in a double-blind study at a maximum dose of 4.8 g/day for 6 months to obese subjects produced no significant differences in weight alteration compared to controls.[89] l-Dopa is thus still another stimulus which, while effective in normal subjects, is ineffective in eliciting growth hormone release in obese subjects.[90,91,9m]

Since insulin levels are elevated in obese subjects, inhibition of insulin release may constitute potential treatment of obesity. Somatostatin inhibits insulin, glucagon and growth hormone release. Therefore, an analog which inhibits insulin secretion but produces no effect on glucagon and growth hormone release could prove useful as an antiobesity agent. Two somatostatin analogs, WY-18092[92] and WY-18-1666[92,93] have recently been reported to significantly inhibit insulin release in rats while having reduced inhibitory action on growth hormone secretion and no effect on glucagon release. In man, both the linear (reduced) and cyclic (oxidized) form of somatostatin appeared to be more potent[94] in suppressing insulin release as compared to glucagon release, and no discernible change in growth hormone levels was observed.

The thyroid hormones T_4 and T_3 continue to be employed in the treatment of obesity[9n,9o] based on the rationale that they increase the dissipation of energy and thereby reduce body weight. However, most of the weight lost consisted of lean body mass (only 1/5 was attributable to loss of adipose tissue),[95] but this ratio could be reversed if dietary nitrogen was increased sufficiently.[96] A physiological dose of T_3 (10 μg) and T_4 (50 μg) produced a greater weight reduction in obese subjects during 4 weeks of fasting than in controls.[9n]

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Section V - Topics in Biology

Editor: T. Y. Shen, Merck & Co., Rahway, New Jersey

Section Editorial

To quote a recently published popular monograph, "Suddenly it is all membranes." Medicinal chemists have been aware of the significance of membrane function and regulation, one way or another, in the course of their study of new drugs for many years. However, recent conceptual as well as technical advances in membrane biology, reminiscent of the golden era of molecular biology in the late 50's and immunology in the late 60's, strongly suggest that closer attention to this fast blossoming field is warranted. Following last year's reviews on the "plasma membrane pathophysiology," "molecular aspects of membrane function" and "ionophores," we continue the examination of membrane related topics: an in-depth treatment of the membrane transport mechanism and a broad survey of potential approaches to novel membrane affectors. In looking for biomedical breakthroughs and novel chemical structures in drug research, biomembrane studies may well provide stimulating new leads.

With an ever-increasing degree of sophistication, many new enzyme inhibitors based on current concepts have emerged. To keep up with this new trend in antimetabolite synthesis, a perspective survey is presented.

Minimizing potential toxicity has become a primary concern in medicinal chemistry, frequently overshadowing the traditional pursuit of potency enhancement. It is encouraging to see that comparative toxicity is gradually being analyzed in terms of biochemical mechanisms and metabolic activation. An awareness of the *in vivo* conversion of drugs to potentially toxic metabolites may help medicinal chemists to select their best leads at an early stage.

The influence of biological rhythms on drug efficacy and toxicity has received much attention in recent years. An introductory chapter on chronopharmacology, the so-called "fourth dimension in drug research," has been included. On a time scale, chronopharmacology is probably still in its infancy or factfinding stage. Nevertheless, it is rapidly moving toward the levels of quantitative analysis and biochemical expressions. Undoubtedly, some medical advantages will derive from the judicial application of this concept in the near future.

Section V - Topics in Biology

Editor: T. Y. Shen, Merck & Co., Inc., Rahway, New Jersey 07065

Chapter 22. Membrane Regulators as Potential New Drugs

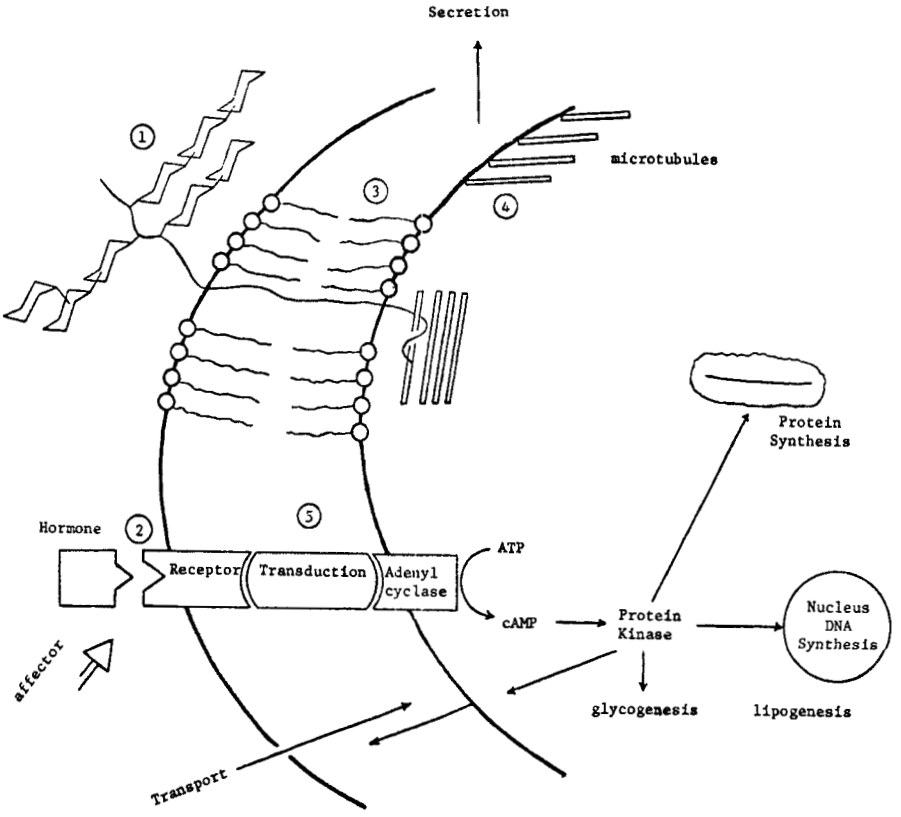
T. Y. Shen, Merck & Co., Inc. Rahway, New Jersey

Introduction - In the last issue of Annual Reports, the broad pathophysiology of plasma membranes[1] and some molecular aspects of membrane function[2] were reviewed. The involvement of specific membrane functions in gamete physiology was also described.[3] A chapter on ionophores and other membrane active antibiotics described a class of chemical agents capable of exerting profound, yet relatively specific, actions on ion transport and related biochemical processes.[4] In the past year the heightened interest in membrane biology has continued at an ever-increasing pace. Several comprehensive monographs,[5-10] reviews, and feature articles[11-16] have appeared in print. As the basic concepts of biomembranes are being substantiated with structural and physical data,[17-19] it becomes timely for medicinal chemists to consider novel and selective membrane affectors as new therapeutic agents. Membranes are obviously important sites for drug intervention because they constitute up to 80-90% of the total cellular mass. Some unique characteristics of biomembranes, such as surface glycoprotein determinants, the bilayer composition and its mesomorphic or liquid crystalline property and complex mechanisms of transport and transduction, have been recognized. To regulate these properties, conceivably novel classes of chemical structures, different from the traditional enzyme inhibitors and antimetabolites which interact mainly with enzyme active sites or protein receptors, would be needed. On the whole, these new agents would exert their metabolic action primarily by interaction with an extracellular site or membrane components, not inside the cell. The basis for their specificity would be the microheterogeneity of cell membranes as demonstrated by the specificity of membrane receptors, surface antigens, cell recognition, cooperation of T and B lymphocytes, etc. In this review a brief survey of target sites in biomembranes for possible chemical intervention, with particular emphasis on the chemical nature of known agents, will be presented. We hope this will provide a basic framework for interested medicinal chemists to formulate specific approaches based on their own insight and new information which is surely to emerge. A specific aspect of membrane research, the liposomes, within the short span of a couple of years, has become a fascinating tool for biological and drug delivery experiments. The current status of this area of active research will also be summarized.

The Membrane Assembly - A schematic presentation of membrane structure and metabolic response to membrane affectors is shown below.

Considering the chemical nature and biochemical properties of various membrane components, one may focus on the following areas as potential sites for drug intervention:

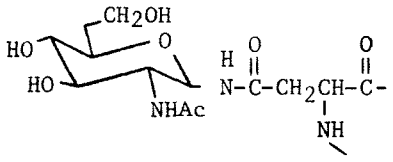
1. Surface determinants and ligand binding sites
2. Receptor regulation
3. Mesomorphic bilayers
4. Submembrane structures
5. Transduction mechanisms



(1) Surface Determinants - The cell surface is a complex and mobile structure that functions in recognizing extracellular materials and in transmitting the interaction or information to subcellular organelles. There are many familiar examples. The release of vasoactive amines and initiation of an atopic asthmatic attack by mast cells follows the specific binding of surface IgE immunoglobulin with certain antigens.[14] The antigen specific receptors of T and B lymphocytes are essential to generating specific immune responses and distributing antigen throughout the lymphoid system.[20] The surfaces of B lymphocytes possess a variety of independent receptors such as IgG, Fc receptors, the constant region of immunoglobulins, and receptors for activated complement components. The latter two types of receptors are also present on the surface of macrophages. Assuming that the glycopeptide juncture Asp-NAcGlc (1) in Fc may be involved in the complement fixation and receptor binding, some analogs have been synthesized as potential inhibitors of the B-cell and macrophage actions.[21]

In addition to surface immunoglobulins, the T lymphocytes carry

other receptors, possibly gangliosides or glycoproteins, which have high affinity for mitogens such as phytohemagglutinin and concanavalin A.[7] These lectins are known to have specificity for carbohydrate determinants such as D-mannose, D-glucose and N-acetyl-D-glucosamine. The mitogenic response of lymphocytes elicited by lectins are readily blocked by saccharide derivatives such as methyl-α-D-mannopyranoside at high concentrations (1mM). The binding regions, which are analogous to that in lysozyme which binds a tetrasaccharide unit of chitotetrose, are likely to recognize an oligosaccharide or glycopeptide sequence; in fact, D-Man-α(1→2)-D-mannose (2) and



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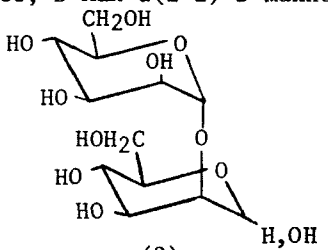
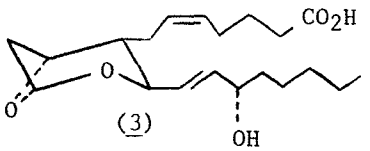


(2)

the trisaccharide derivative have been found to be 6 to 25 times more effective as mitogenic inhibitors.[22] The binding of macrophages with the migratory inhibitory factor (MIF) was reported to involve fucose in the MIF glycoprotein.[23] Conceivably some fucose derivatives may inhibit macrophage functions. Other cellular systems such as tumor cells, fat cells and platelets also possess lectin binding sites and respond to interaction with Concanavalin A (Con A), etc. Interestingly, Con A and wheat germ agglutinin not only block the insulin effect on fat cells but initiate an insulin-like metabolic effect in terms of glucose utilization, epinephrine-stimulated lipolysis, etc.[24] Lectins also affect platelet aggregation and the release of mediators.[5] The roles of saccharides in these effects remain to be clarified. Whether prostaglandins and thromboxanes (3)[26] are involved in their actions remains unknown.



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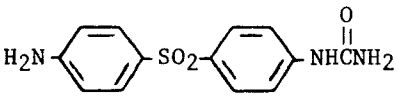


(4)

The saccharide binding site on cell surfaces may also be involved in intercellular adhesion.[27,28] Some glycosyltransferases which incorporate activated sugars into glycoprotein or glycolipids are associated with cell membrane. These enzymes may bind extracellular saccharides, or saccharide determinants of another cell. The activity of glycosyltransferases has been shown to vary with the metabolic states of cells.[29,30] For instance, a reduced level of activity, resulting in an incomplete synthesis of saccharide determinants, was found with transformed cells. Thus, inhibition or stimulation of glycosyltransferases may indirectly alter membrane characteristics and interfere with cellular interactions and influence cytoplasmic metabolism.

The effective use of determinant analogs to inhibit receptor-deter-

minant interaction depends greatly upon an intimate knowledge of the chemical structures involved in the binding. With saccharide determinants, the limiting factor at the present is their structure elucidation. Forty-four disaccharide sequences have been identified as naturally occurring sequences.[31] The diversity of these structures would provide a basis for specificity of disaccharide derivatives as therapeutic agents once the correct sequence is elucidated.

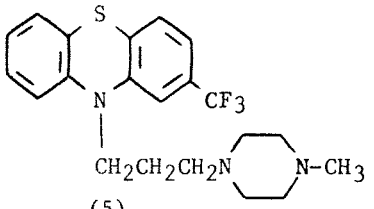
In infections with protozoan and metazoan parasites, the membrane antigens are initially recognized by the host immune mechanism. However, through an active process of antigenic variation, the surface glycopeptide and glycolipids are extensively modified to escape the T-cell recognition process. As a result, an integrated existence with their hosts is established.[32] Conceivably any interference with the biosynthesis of new membrane structure, e.g., by schistosome tegument during the first few hours after penetration, would abort the infection.

In this regard, it is of interest to note that AUS (MK-241, 4), a chemoprophylactic agent for Marek's Disease,[33] was shown to inhibit the incorporation of choline to membrane phosphatidylcholine.[34] Marek's Disease is a lymphoproliferative avian leukosis of major economic importance to the poultry industry. It is characterized by virus induced cell transformation, membrane changes and immunosuppression.[35,36] The mode of action of AUS may be attributable to its membrane actions.

(2) Receptor Regulation - The inhibition or stimulation of membrane receptors by analogs of hormones or mediators is beyond the scope of this review. Suffice it to mention that the function of membrane receptors, as well as membrane enzymes, e.g., Na^+K^+ AtPase and acetylcholinesterase, can be modulated by agents which cause local conformational changes either by direct perturbation or by alteration of the composition of membrane structures.[37] The discovery of allosteric inhibitors of enzymes is often hampered by the lack of detailed structural information. In the case of membrane enzymes and receptors, the multicomponent organization of the membrane assembly may respond to a greater variety of chemical agents such as sterols, fatty acid and phospholipid derivatives. Alternatively, the high turnover rate of some membrane constituents renders them more susceptible to biosynthesis inhibitors.

Amphiphilic cations such as trifluoperazine (5) and chloropromazine (6) stabilize the erythrocyte membrane at 10^{-6}M.[38] They also increase de novo synthesis of phosphatidyl inositol in both the lymphocytes and rat liver systems, possibly owing to inhibition of phosphatidate phosphohydrolase and glycerophosphate acyl transferase.[39] A rapid increase in phosphatidyl inositol turnover occurs in lymphocytes activated by phytohemagglutinin or other mitogenic stimuli. The inhibition of this process, analogous to the inhibition by AUS of Marek's virus induced membrane biosynthesis cited above, may exert immunoregulatory effects also.

An interesting concept of self regulation of membrane receptors has recently been pointed out.[40] The concentration of a ligand can regulate



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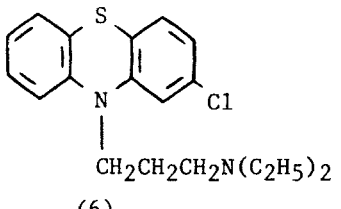
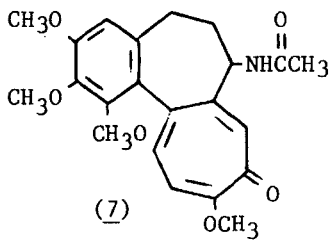


(6)

the concentration and/or binding properties of its own receptor on the surface of target cells. The formation of an antibody-antigen complex on the surface of leukemic or B-lymphocytes is followed by pinocytosis and disappearance of the surface antigen of Ig molecule. This process of "antigenic modulation" is a mechanism which enables leukemic cells to escape from the host's immune response. Similar disappearance of surface-receptors has been observed with insulin, growth hormone and thyrotropin-releasing hormone (TRH). Receptor regulation by ligands has been extended to β-adrenergic receptors. The self regulation of neurotransmitters also varies with a circadian periodicity. Interestingly, the modulation of these receptors by β-adrenergic agonists is prevented by the antagonist, propanolol.

Colchicine (7) has been shown to inhibit the internalization of lectin binding sites of polymorphonuclear leukocytes.[41] Local anesthetics also affect the distribution of surface receptors[42] (see below). As receptor modulation may be involves in many cases of tolerance or tachyphylaxis induced by chronic exposure to high concentrations of hormones, neurotransmitters and drugs, it would be of interest to uncover new membrane agents affecting this process.



(7)

(3) Mesomorphic Bilayers - The fluidity of the bilayer membrane is largely determined by the composition of membrane lipids. The heterogeneity of the lipid phase leads to regional differences in fluidity. The polar head groups of the phospholipid molecules are involved in the lipid organization. Sterols interact selectively with the phospholipid chains, reducing its molecular area and conferring rigidity.[43] Thus, membrane fluidity is proportional to the ratios of double bond index/saturation and phospholipid/cholesterol. Most cells seem to be able to incorporate exogenous lipids. In some cases the process is greatly facilitated by a family of lipid exchange proteins. Replacement of membrane fatty acid has been shown to affect the growth and lectin-induced agglutinability.[43] Artificial insertion of free fatty acid and their simple derivatives, e.g., glyceryl mono olein, into biological membranes have inhibited secretory granules, enhanced cell fusion and protected hemolysis of erythrocytes. Fatty acids are also membrane modulators which enhance activation of erythrocyte adenylate cyclase by catecholamines.[44] Unsaturated fatty acid with long chains and cis double bonds are most effective in disrupting the orderly arrangement of fatty acyl side-chains in the bilayer by virtue of their rigid geometry and ability to increase fluidity and lower melting point. Palmitoleic (Δ

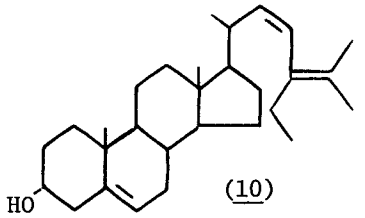
9-10 C16:1c) oleic (Δ 9-10 C18:1c), cis-vaccenic (Δ 11-12 C18:1c) and eicosenoic (Δ 11-12 C20:1c) acids were much more effective than related saturated analogs or trans isomers in enhancing the activation of adenylate cyclase by isoproterenol. These acids apparently act on the guanyl nucleotide site which regulates the adenylate cyclase system.

Conversion of the cis double bond in fatty acids to a cyclopropane ring by carbene addition removed the oxygen sensitivity of the former but preserved the membrane perturbation effect.[45] The activation of lymphocytes by antigens and lectin is associated with an increase of membrane *mobility*, readily measured by the movement and cap formation of surface receptors. The cap formation was facilitated by alkoxy esters of these cyclopropyl acids. Among a small group of esters tested, compound (8) was more active and less cytotoxic than its analog (9). Conversely, the cap formation or lymphocyte activation, was markedly suppressed by enrichment with exogenous cholesterol which increases the microviscosity of lymphocyte membrane.[46]

$CH_3(CH_2)_7\Delta(CH_2)_7CO_2R$ R = $(CH_2CH_2O)_2CH_3$ (8)

$(CH_2CH_2O)_3CH_3$ (9)

Another example of allosteric regulation by modification of the membrane steroid is the effect on erythrocyte glucose transport.[47] Removal of cholesterol from the membrane inhibited glucose transport. The restoration of activity required replacement of cholesterol by 3-β-hydroxy, but not 3-keto steroids. Stigmasterol (10), ergosterol (11), and 7-dehydrocholesterol were particularly effective, requiring only 2%, 6% and 10% replacements, respectively. Apparently, a more planar nucleus and a more bulky side-chain than cholesterol also aided in membrane restoration. Exogenous ergosterol was also very effective in replacing tetrahymanol (12) in the membrane of the ciliated protozoan, tetrahymena pyriformis.[48] The replacement induced a profound alteration of the phospholipid class composition, a marked increase of phosphatidylethanolamine, as well as changes in their fatty acyl chains.



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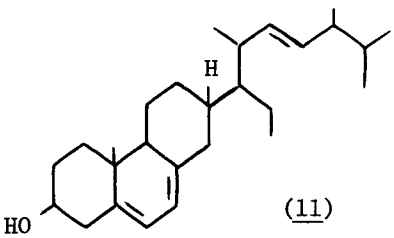

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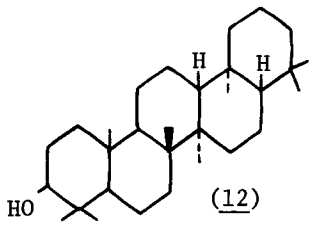


(12)

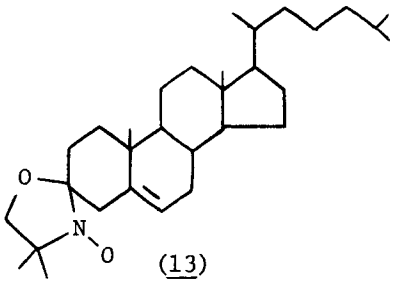
The membrane fluidity also changes with the metabolic state of the cell. It is greater for transformed tumor cells and sensitized lymphocytes than normal cells. It is also subject to hormonal actions. Cortisol enhances and insulin decreases fluidity. Although fluidity per se is not a precise parameter to measure drug actions, localized fluidity change may provide an effective and indirect way to regulate membrane functions and associated enzymes. The effects of cortisol and insulin on erythrocyte membrane enzymes have been measured in terms of changes of their Hill coefficients.[37,49]

Membrane fusion between lysosomes and phagosomes is a crucial step for such normal cell functions as phagocytosis, LDL metabolism,[50] and immunologic defense. On the other hand, the hydrolytic enzymes in lysosomes, once released outside the cell, are injurious to surrounding tissues. The membrane stabilizing effect of corticosteroids and, to a lesser degree, of various nonsteroidal anti-inflammatory agents, serve to inhibit the tissue degenerative effect of lysosomal enzymes. The intensive search for more effective lysosome stabilizing agents in the past decade has not been very fruitful. In the related study of erythrocyte membrane stabilizers, a number of pharmacological agents, tranquilizers, antihistamines, local anesthetics, polyene antibiotics, and ionophores were examined. The direct application of membrane stabilizers as anti-inflammatory agents is often complicated by their other physiological actions.

The influence of membrane composition and fluidity on Ca^{++} transport of sarcoplasmic reticulum membrane and possible correlation with hereditary muscular dystrophies have also been discussed.[37]

There is increasing evidence that both the proteins and the phospholipids of membranes are asymmetrically distributed in the two halves of the bilayer. For example, phosphatidylserine which bears a net negative charge at physiological pH, concentrates mainly in the cytoplasma half of the membrane. Thus, the two halves of the closed membrane bilayer may respond differently to various drug induced perturbations. A bilayer couple theory was proposed to explain the interaction of these amphipathic drugs with human erythrocytes.[51] Anionic drugs, e.g., chloropromazine and lidocaine, intercalate mainly into the lipid in the exterior half of bilayer and cause a differential expansion in that layer, while permeable cationic drugs are attracted by the negative field of the cytoplasmic layer.

Nitroxide labeled lipids, e.g., derivatives of cholesterol (13) and stearate (14), are used widely as ESR spin probes. It is of interest to note that the nitroxide group is also a potent perturbant in focal regions of cell membrane, sometimes leading to irreversible changes.[52] As photosensitive labels, a variety of fatty acids containing an azido group in different positions in the alkyl chains (e.g., 15 and 16) were synthesized recently from the readily available hydroxy acids.[53] Several of these have been incorporated by E. coli into membrane phospholipids. Photolysis-induced cross-linking of the fatty acids to the structures in their immediate vicinity should shed further light on phospholipid-protein interactions in the bilayer.[54]



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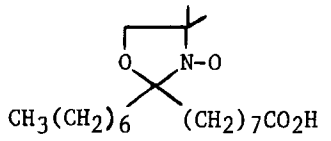

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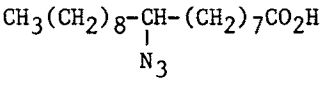

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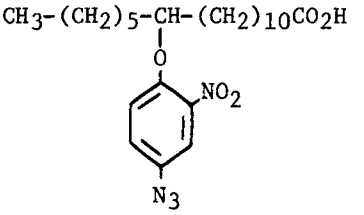

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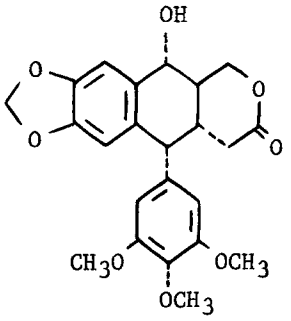
(4) Submembrane Structures and Transduction Mechanisms - The intracellular fibrillar structures are composed of microtubules (MT) and microfilaments (MF). MT are large, rigid, cylindrical molecules built from a protein subunit called tubulin. The biology of cytoplasma microtubules was reviewed recently.[8,11] Cytoplasmic MT are usually in a state of rapid assembly and disassembly, probably involving Ca^{++} and cyclonucleotides. The assembly or polymerization is inhibited by colchicine (7), podophyllotoxin (17) and vinblastine which bind with high affinity to fibrous tubulin protein within the cell.[11,55] The antitumor ansamacrolide maytansine also binds tubulin competitively with vincristine with a k_i-value of 0.13 μM.[56]

Mescaline (18), a catecholamine-like neurotransmitter analog also having the trimethoxyphenyl partial structure of colchicine, was recently shown to bind purified microtubule protein.[57] Like colchicine, it is a mitotic inhibitor and it inhibits the assembly of tubulin subunits. Its metabolite, N-acetyl-mescaline is a moderate mitosis inhibitor. This finding raises the possibility that neurotransmitters and their metabolites, in addition to their effect on cell membranes, may regulate the polymerization state of fibrous protein within the cell.



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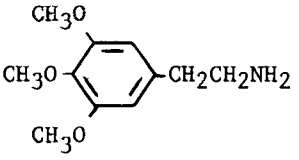
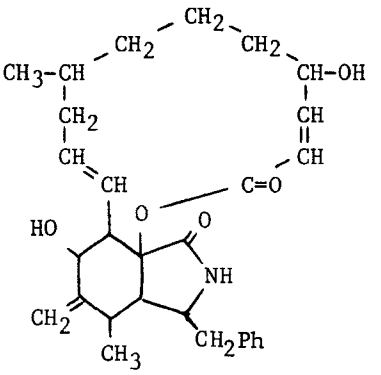


(18)

MT control the membrane protein movement indirectly through microfilaments. MF are made of actin-like subunits and are sensitive to cytochalasin B (19). Both MT and MF contribute to the transmembrane control of surface phenomena such as immunoglobulin capping and receptor mobility. MT "anchor" receptors and limit their mobility[58] whereas MF may redistribute receptor through its contractile action. They may act synergistically or in an opposing manner. The combined treatment with colchicine and cytochalasin B was shown to be more effective in regulating receptor mobility in several systems than either one alone.[42]



(19)

In addition to their effects on membrane lipids, local anesthetics impair both MT and MF. Lidocaine inhibits the polymerization of MT by competing with Ca^{++}.[42] Calcium ion transport and the increased level of cGMP both play prominent roles in the stimulation of lymphocytes by mitogens.[59,60] cGMP is elevated by phorbol ester and enhances redistribution or receptor mobility. An early event in the commitment of lymphocytes to mitogenesis is apparently blocked by colchicine.[60] It was concluded that the modulation of lymphocyte mitogenesis after interaction with mitogens, e.g., lectins, is an indirect result of the alteration in MT and MF assemblies. Cytochalasin B (19) has been reported to inhibit cytokinins, cell locomotion and phagocytosis by macrophages.[62] Partial selectivity of cytochalasins toward different cells has been demonstrated. We have found that the effect of cytochalasin derivatives on mixed lymphocyte reaction is not parallel with their cytotoxicity. The synthesis of cytochalasins and their analogs, currently going on in several laboratories, may demonstrate the therapeutic potential of this class of MF inhibitors.

The mechanism of membrane transduction, i.e., the activation of adenylate cyclase or other metabolic responses after the binding of a ligand with its membrane receptor, remains a major challenge in biomembrane research. In addition to the possible roles of MT, MF, Ca^{++} and cyclonucleotides, the oxidation of sulfhydryl groups, and the translocation of an "*active*" subunit of cholera toxin[63] and thyrotropin[64] within the membrane domain to activate adenylate cyclase have been postulated. The biochemical relationship of different receptors, e.g., the coupling of prostaglandin E_1 receptor with acetylcholine receptor,[65] and the reciprocal coupling of cGMP and cAMP levels in some cases,[66] introduced another level of complexicity. Undoubtedly, much information will be forthcoming in the next few years.

Liposomes - The use of liposomes as carriers of drugs and enzymes for therapeutic applications has received a great deal of attention recently. Liposomes are synthetic vesicles, either unilamellar or multilamellar, prepared from phospholipid dispersions in aqueous media by ultrasonication. They have a diameter of 200-400Å and are capable of entrapping, or microencapsulating, solutes in the medium, such as drugs, immunoglobulins or virus vac-

cine. Liposomes are well tolerated when given parenterally. Upon delivery to target tissue the enclosed active ingredient is released by fusion or by endocytosis to exert its action locally. Some success to reduce the toxicity of drugs such as adriamycin has been reported.[67] It has been used to prolong the duration of intramuscular absorption of insulin, cefazolin, etc.[68] The rate of release is regulated by the amount of cholesterol incorporation. The distribution, cellular affinity and metabolism of liposomes are largely determined by the membrane composition of the vesicle, especially the surface determinants. For instance, liposomes with specific antifibroblast or anti-HeLa immunoglobulins incorporated into the vesicle have shown specific affinity for fibroblast and HeLa cells, respectively.[69] Liposomes with exposed galactose residues also showed enhanced hepatic uptake. Most recently, an enzyme, hexosaminidase A, was introduced into Tay-Sachs polymorphonuclear leukocytes by means of immunoglobulin-coated liposomes.[70] The vesicle protects the enzyme from immune recognition sites and metabolic destruction in vivo. The surface immunoglobulin interacts with cell surface Fc receptors. The uptake of the enzyme was demonstrated by cytochalasin B which prevents phagocytosis but not surface adherence. It is interesting to note the influence of surface charges on the in vivo distribution of these vesicles: negative charges favor uptake by spleen and marrow, positive charges direct liposomes to lungs and neutral liposomes are mostly taken up by the liver.[71,72] Understandably, the immunological adjuvant effect of liposomes was highest with negatively charged ones.[73]

Liposomes have been claimed to facilitate the oral absorption of insulin resulting in significant reduction of blood glucose levels in diabetic rats.[74] However, since liposomes are not expected to remain intact in the gastrointestinal tract, further clarification of the nature of this apparent uptake of insulin is awaited.[75]

In summary, liposomes represent a biological approach to selective drug delivery which may have considerable promise.

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Chapter 23. Some Features of Solute Active Transport Across Biological Membranes

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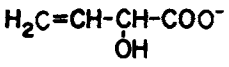
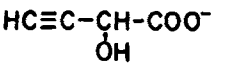
This review will focus on recent developments in membrane biochemistry and biology which have begun to develop molecular insights into how solute molecules are transported across biological membranes. Much of the recent advance comes from experiments with bacterial cells where homogeneous cell populations, the ready availability of defined mutants, and the use of isolated membrane vesicles have aided investigation, but animal cell transport has also received attention. A variety of recent books and review articles are available,[1-14] and Chapters in last year's volume 10 dealt with aspects of plasma membrane pathophysiology[15] and molecular aspects of membrane function.[16]

Active Transport - In passage of a molecule across a biological membrane one can distinguish free passive diffusion (i.e., water movement), facilitated diffusion, and active transport. The latter two processes show specificity and saturation kinetics (attainment of a finite maximal velocity), indicating mediation by specific membrane carrier proteins. Facilitated diffusion involves passage of a solute down its electrochemical gradient and requires no energy input; this is exemplified by glucose transport from blood into erythrocytes. Active transport is concentration of solute against a gradient and occurs for example in amino and sugar transport in kidney and intestinal epithelial cells and is highly developed in bacteria and other free living unicellular organisms. We shall concentrate on active transport which is a process requiring at least two distinct components: (1) energy-coupling mechanisms; (2) solute-specific membrane carrier. Typical concentration gradients may be 10^6 for phosphate transport in yeast, 10^7 for H^+ between gastric epithelium and stomach lumen, and 5×10^2 for amino acid and sugar solutes in isolated bacterial membrane vesicles.[12]

Group Translocation - One mechanism for unidirectional uptake is the covalent modification of solute during passage through the membrane. This is not strictly active transport but has the same effect of concentrating molecules inside the cell. The most well-studied example is phosphorylation of such hexoses as glucose, fructose, and mannose during concentrative uptake in anaerobic and facultatively aerobic bacteria.[17-19] The phosphoryl donor is phosphoenolpyruvate (PEP) and for each molecule of sugar transported, one molecule of PEP is hydrolyzed to pyruvate. Two peripheral membrane enzymes, Enz I and HPr, are sequentially phosphorylated as reaction intermediates before interaction with and phosphoryl transfer to the hexose bound to two integral membrane protein components of this phosphotransferase system (PTS). The PTS system is still functional in isolated cytoplasmic membrane vesicles[20] of bacteria.[21] These vesicles also have membrane-bound flavoenzymes such as D- and L-lactate dehydrogenases.[12] Addition of 2-hydroxy-3-butenoate (vinylglycolate)[22] or 2-hydroxy-3-butynoate[23] results in oxidation by these dehydrogenases



vinylglycolate



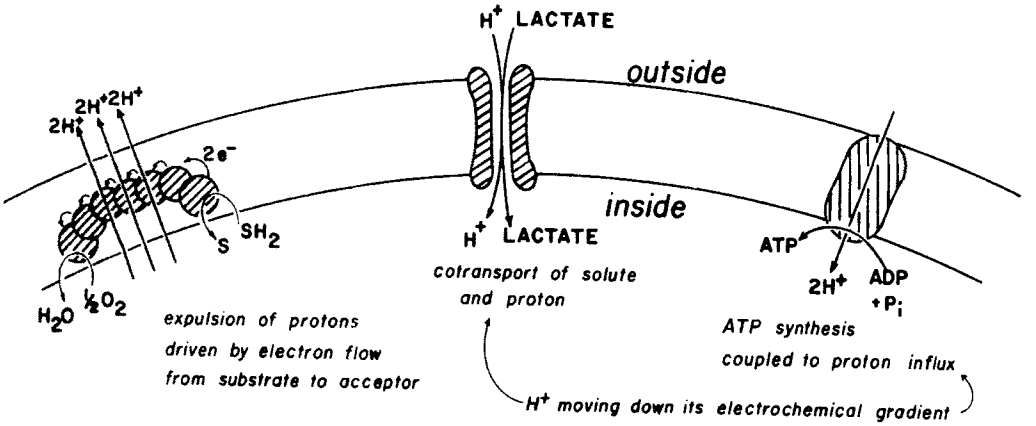
hydroxybutynoate

to the olefinic and acetylenic keto acids respectively. These products label the Enzyme I component of the hexose phosphotransferase system specifically and cause blockade of sugar uptake.[24] Since the PTS is absent from eucaryotic cells this approach suggests a potentially useful approach to bacterial transport blockade. The inactivation persists in bacterial whole cells also.[25] Inactivation by the acetylenic keto acid but not the olefinic keto acid is reversible with thiols.[26]

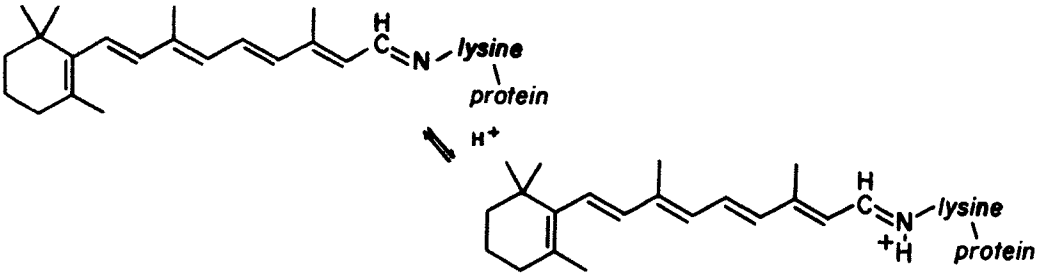
In obligately aerobic bacteria the PTS system is absent; in Azotobacter vinelandii glucose is actively transported unmodified and is refractory to vinylglycolate inhibition. In Pseudomonas aeruginosa, glucose is transported by an inducible system,[27] either as free glucose, or, after oxidation by membraneous glucose dehydrogenase, as gluconate.[28] Other examples of enzyme-catalyzed modification of solutes during transmembranal passage include testosterone oxidation to androstenedione during transport in Pseudomonas testosteroni,[29] adenine conversion to AMP,[30] and conversion of butyrate to the thiolester butyryl CoA by a CoA transferase.[31]

The Chemiosmotic Hypothesis - One of the central questions in solute active transport where solute molecules are not chemically modified is how is this thermodynamically unfavorable process driven. How is the energy, either from ATP hydrolysis by membrane ATPases[32] or by electrons flowing down a series of controlled potential drops in the membrane respiratory chain, transduced to drive solute accumulations? What is the nature of the energy-rich membrane state? One hypothesis can be described as a conformational coupling one where electron flow from oxidizable substrate to O_2 in the membrane leaves proteins in high energy conformational states which can relax back to their low energy conformations, with relaxation delivering the energy to do work. In the chemiosmotic hypothesis of Mitchell[33-36] the key element is vectorial metabolism, that electron flow down the membrane cytochrome chain leads to unidirectional translocation of protons from inside to outside, an electrogenic pumping of protons, setting up an electrochemical gradient of H^+. Given the apparently valid assumption that the membrane is essentially impermeable to free diffusion of protons, the electrochemical potential can do work. The term proton motive force (ΔP) is used to describe this potential which has a chemical potential component, which for the proton is simply ΔpH, and an electrical potential component $\Delta\psi$. $\Delta P = \Delta\psi + \Delta pH$ (in millivolts). Once a net negative interior electrochemical potential has been set up, if the specific transport carrier proteins translocate specific solutes from outside to inside the membrane barrier along with (i.e., symport) protons then this influx of protons will dissipate the electrochemical gradient concommitant with

active transport. A proton circulation would arise--pumping out as a consequence of respiratory chain action and ingress coupled to solute uptake.[33-36]

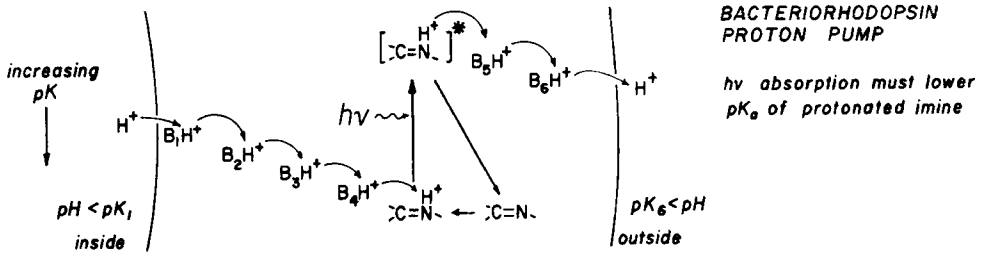


A key experimental verification for chemiosmosis arises from successful attempts to measure membrane potentials using the distribution of lipid soluble anions and cations across the membrane[35] and subsequent Nernst calculations to obtain $\Delta\psi$. In S. faecalis cells $\Delta\psi$ is -150 to -200 mV on the interior[37,38] and about 100 mV, interior negative in membrane vesicles of E. coli[39,40] which are allowed to oxidize various exogeneous substrates. Even more convincing that a transmembrane potential is a primary force for powering active transport are observations that a potential generated by an artificially induced diffusion gradient of K^+ (in the presence of valinomycin) independent of any substrate oxidation, will drive solute uptake transiently.[41,42] Also of note is the purple membrane protein, bacteriorhodopsin, of extremely halophilic bacteria as biological energy transducer.[43] The purified protein has a molecular weight of about 35,000, and is more than 70% α-helix with seven α-helical arrays, some of them spanning the purple membrane. This is the only protein in purple membrane patches where it is packed in an oriented hexagonal array. Illumination of bacteriorhodopsin in a synthetic planar phospholipid bilayer produced a $\Delta\psi$ of about 50 mV.[44] It may be that protons are translocated from the inside face of the membrane to outside by anisotropic protonation and deprotonation of the imine linkage of the retinal chromophore bound to an ε-amino of a lysyl residue of the apobacteriorhodopsin.



retinal in imine linkage in bacteriorhodopsin

Using pure bacteriorhodopsin, mitochondrial ATPase and hydrophobic mitochondrial coupling proteins in synthetic phospholipid vesicles, it could be shown that illumination led to ATP synthesis from ADP and Pi.[45] With the pure bacteriorhodopsin it is possible to demonstrate a cycle of bleaching and recoloration within milliseconds, with proton release and uptake in discrete phases,[46] supporting the argument that this protein may be a physiological photoreceptor in the membrane. A hypothetical scheme showing how bacteriorhodopsin, immobilized within the middle of purple membrane patches, might act as a proton pump is indicated below.[46]



Recent experiments in membrane vesicles derived from kidney epithelial cells indicated a transmembrane potential could be generated and used to power glucose active transport.[47] Finally, it has been shown in isolated bacterial membrane vesicles, using a flow dialysis technique that both a ΔpH component and a $\Delta\psi$ component of the electrochemical potential are separately measurable. Some active transport systems (e.g. glucose-6-phosphate, lactate, succinate, lysine) respond to ΔpH; some (proline, serine, cysteine) respond to $\Delta\psi$; yet others respond to the total driving force ΔP (lactose, glycine, tyrosine).[47a]

ATPase Structure and Function - Much recent progress has been made on membrane ATPase structure[32] and function. The mitochondrial and chloroplast ATPases are hypothesized to act, like the carrier proteins, as proton conductors from outside to inside during oxidative phosphorylation and ATP synthesis; the influx of protons to neutralize the electrochemical potential is posited to drive ATP formation. In the reverse, ATPase mode, protons are pumped from inside to outside to set up the proton motive force required for work such as solute transport. The bacterial membrane ATPase of E. coli[48,49] and a thermophile SP3[50] resembles the mitochondrial enzyme in possessing 5 subunit polypeptides of differing size: α, β, γ, δ, ε. The δ-subunit of the solubilized Ca^{++}, Mg^{++}-dependent ATPase is specifically required for reassociation to membranes.[49] Studies with a coli mutant DL-54 indicated that non-functional enzyme was present in isolated membrane vesicles which could not transport proline or lactose normally.[51] Dicyclohexylcarbodiimide (DCC), a specific inhibitor of functional ATPase, restored the ability of the mutant vesicles to carry

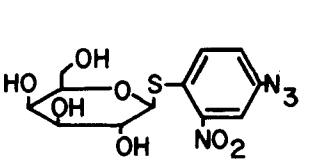
out active transport. It was found that DL-54 vesicles could not maintain a $\Delta\psi$ across the membranes but addition of DCC allowed buildup of a detectable membrane-potential.[52] It could be shown that the mutant ATPase had a structural defect making membranes leaky to protons; DCC blocked this proton leak, apparently by covalent modification of a membrane protein, possibly an ATPase subunit. ^{14}C-DCC labels a 9000 mw polypeptide in the membrane.[53] DCC treatment similarly restores functional integrity to membrane vesicles that have become specifically permeable to protons after exposure to chaotropes such as guanidine or urea.[54] Experiments with mitochondrial ATPase in liposomes have shown that an electrical potential of about -210 mV is required ($\sim$ -7 Kcal/mole) before detectable ATP synthesis is driven.[55]

In animal cells the Na^+, K^+-dependent ATPase in the plasma membrane is responsible for the Na^+, K^+ pump of these cells, coupled active transport of Na^+ out and K^+ in.[56] The purified enzyme from several sources has a large and a small subunit[57-60]; the large subunit has the active site residue which is covalently phosphorylated during catalysis and the binding site for the specific inhibitory cardiac glycosides[58,61] (e.g. ouabin, strophanthidin). The cardiac glycosides inhibit ATPase action and the pumping of Na^+ out and K^+ in specifically from the outside face of sealed erythrocyte ghosts and not when on the inside. The nature of the amino acids forming the glycoside binding site are unknown. The small subunit, without an as yet ascribed function, is a glycoprotein and is probably exposed at the membrane outer surface.[59] The large chain spans the membrane and Kyte has suggested that sodium and potassium ions move through the membrane via a channel down the center of the ATPase,[62,63] invoking conformational changes to couple ATP hydrolysis to specific vectorial passage of the cations. When purified canine renal red medulla ATPase is reconstituted with sealed phospholipid vesicles there is active pumping of Na^+ and Cl^- but not K^+[64]; canine brain enzyme[65] and enzyme from shark rectal gland[66] in synthetic liposomes do show ATP-dependent pumping of both Na^+ and K^+ vectorially.

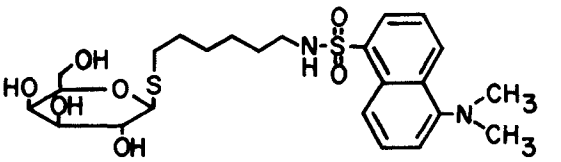
A structurally similar Ca^{++}-dependent ATPase is involved in the intracellular pumping of Ca^{++} from cytoplasm into sarcoplasmic reticulum in regulation of muscle cell contraction. The reticulum Ca^{++} binding proteins[67] and the ATPase[68,69] have been purified and characterized and reconstitution of a Ca^{++} pump in phospholipid vesicles has been achieved.[70] The active sites of the Na,K-ATPase and Ca^{++}-ATPase, both phosphorylated during catalysis, appear similar[66] and thus form a class structurally and perhaps catalytically distinct from the mitochondrial, chloroplast and bacterial ATPases noted above (where it is protons that may be specifically passed through these latter enzymes).

Carrier Proteins - Turning from the energy coupling factors to the membrane carrier proteins, it appears that in bacteria there may be two classes of proteins to consider[11]: (1) the periplasmic binding proteins which are releasable by osmotic shock and absent from subsequently isolated membrane vesicles[72,73] and; (2) a second class of proteins which are resistant to osmotic shock, are integral membrane proteins and are the

carrier proteins present in isolated membrane vesicles.[12] The latter category clearly comprise membrane carrier proteins functional in transport and due to their difficulty in solubilization and purification rather little is known about them structurally. The classic example is the lactose carrier protein identified in inactive form after alkylation with N-ethylmaleimide.[74] Present in membrane vesicles,[75] it has recently been labeled therein by photolyzable lactose analogue, p-nitrophenylazido galactoside.[76,77] Recent experiments with dansylaminohexylthiogalactoside,[78,79] a competitive inhibitor for lactose binding to the carrier protein, but one which is not transported across the membrane, show the exciting finding that the lactose carrier protein is functionally cryptic (i.e. either physically inaccessible to external carrier or accessible in a low affinity conformation) in the absence of an electrochemical potential across the membrane. Using bacterial mutants with controlled mem-



p-nitrophenyl azido-galactoside



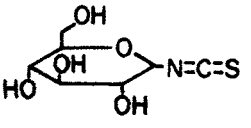
dansylaminohexylthiogalactoside

brane lipid content, it has been argued that membrane fluidity and fatty acyl melting temperatures affect rates of lactose transport.[74a] Recently progress has been made in the solubilization and partial purification in the presence of non-ionic detergents of a succinate binding protein[74b] from E. coli and a folate binding protein[74c] from a lactobacillus.

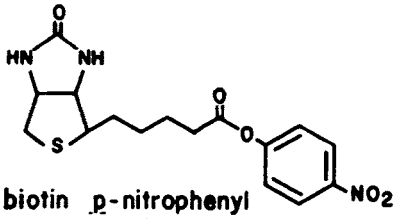
On the other hand, the periplasmic binding proteins which are hydrophilic are easily purified and studied. Transport systems sensitive to osmotic shock for which periplasmic binding proteins are isolable include ions such as $SO_4^=$, $PO_4^=$, vitamins such as thiamin, riboflavin, cyanocobalamin, a variety of amino acids and sugars.[11] Genetic evidence indicates coinduction of binding proteins and transport competence, and mutants have been found which revert to transport positive on regain of functional binding proteins. For the histidine transport system there is good evidence for both an integral membrane carrier and a periplasmic protein for efficient histidine active transport.[80,81] It may be that these water-soluble periplasmic proteins are preconcentration devices for interaction with membrane bound carriers. Attempts at reassociation of purified binding proteins with membranes have not succeeded reproducibly.[11] Genetic evidence exists that one of the components of the β-methylgalactoside transport system may be identical with the bacterial chemoreceptor for galactose.[82] It has been argued without molecular details that shock-sensitive active transport systems may be energized preferentially by ATP hydrolysis while shock-resistant systems are powered by electron flow down respiratory chains.[83]

Transport Inhibitors - A number of compounds, some noted in the above paragraphs, have recently been reported to act by specific inhibition of

some membrane transport process (as opposed to an effect on cell membrane synthesis, e.g., antibiotics such as penicillins and cycloserine, or membrane structural integrity, e.g., polyene antibiotics). Classical uncouplers of respiration such as dinitrophenol and CCCP (carbonylcyanide-m-chlorophenylhydrazone) are thought to act as lipophilic weak acids, by equilibrating protons across the membrane and dissipating the membrane potential.[36] Cardiac glycosides block Na,K-ATPase action.[56] Cytochalasin B has been used as a specific inhibitor of the erythrocyte glucose carrier[84] and a preliminary report claimed some specificity with glucosyl isothiocyanate.[85] Biotinyl p-nitrophenyl ester was reported as an affin-



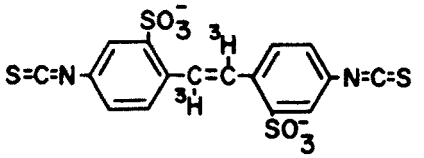
isothiocyanoglucoside



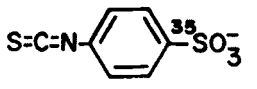
biotin p-nitrophenyl ester

ity label for biotin transport in yeast,[86] but the inactivation was labile to work-up.[87] Certain of the Colicins, proteins elaborated by some bacteria which kill others, are thought to interfere with active transport pleotropically.[88,89] D-chloroalanine is oxidized by E. coli membrane D-alanine dehydrogenase to chloropyruvate an in situ alkylator which uncouples the energized membrane state from use by a variety of membrane carriers.[90-92]

Little is known about the nature of transport carriers in animal cells. In the erythrocyte membrane anions are transported by facilitated diffusion and irreversible inactivation by affinity labels (4,4'-diisothiocyano-2-2' [^{3}H] stilbenedisulfonate,[93] 1-isothiocyanate-4-benzenesulfonate[94]) have resulted in labeling of a protein of 100,000 molecular



4,4'-diisothiocyano-2,2'-stilbene-disulfonate

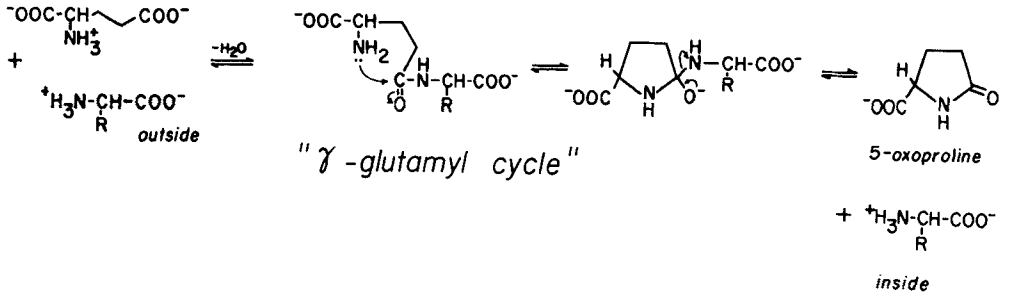


1-isothiocyanate-4-benzenesulfonate

weight, presumably the anion transport protein. The glucose carrier protein, catalyzing facilitated diffusional entry of glucose, has been solubilized from erythrocytes with detergent. On detergent removal and incorporation of the crude 100,000 MW protein fraction into liposomes, D-glucose but not L-glucose can now pass into the liposomes[94a].

It has been argued that sucrase, isomaltase may be involved in intestinal disaccharide uptake.[95,96] Meister has postulated, with no direct evidence, that amino acid transport in kidney epithelia may occur by conversion of exogeneous α-amino acid to a γ-glutamyl-α-amino acid via a

membraneous γ-glutamyl transpeptidase. Intramolecular breakdown to the liberated (and internalized?) amino acid and the cyclic 5-oxo-proline would be catalyzed by the γ-glutamyl cyclotransferase in this so-called "γ-glutamyl cycle".[97]



One area of clear interest is in regulation of ion and metabolite transport both in normal and diseased states. The stimulatory effect of β-adrenergic catecholamines on potassium uptake has been examined in turkey erythrocytes and involves activation of plasma membrane adenyl cyclase with eventual modulation of membrane ATPase activity.[98] There is evidence for increased transport of sugars[99] and amino acids[100] in virally transformed fibroblasts although the meaning of these changes is not understood since glucose transport normally increases with increased rates of cell growth.[101] Transport phenomena clearly affect drug delivery since some tumors which become resistant to methotrexate do so for lack of transport[102,103] while chloroquine[104] is actively transported into erythrocytes.

Unfortunately in a review of this brevity space is not available to delve into tangental but important topics such as fluidity and microheterogeneity in membranes,[105] lateral mobility[106,107] and the transmembrane nature of membrane proteins,[108] lectin receptors,[109] hormone receptors, membrane biosynthesis and assembly,[111,112] and changes in membrane structural components in development[113] or on transformation[114] but the indicated references are entries into the recent literature.

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Chapter 24. The Antimetabolite Concept in Drug Design

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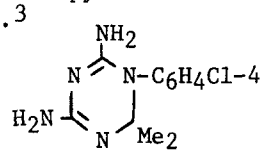
For all practical purposes, recognition of the possibilities of antimetabolites as drugs dates from D. D. Woods' discovery in 1940 that the effectiveness of sulfanilamide is due to its antagonism to p-aminobenzoic acid (PABA), a bacterial growth factor.[1] The concept has widened considerably since then, with recognition of biochemical strategies other than growth factor antagonism. Today the term antimetabolite encompasses a multitude of compounds which inhibit the normal operations of enzymes, transport and binding proteins and receptors, by substitution for their usual substrates or cofactors and regulatory agents.

Medicinal chemists were impressed by the success of the sulfa drugs and the accompanying theory, but became disenchanted when further applications met with persistent failure. Their skepticism was akin to the distrust entertained toward "electron-pushers" by the previous generation of organic chemists. The experience in the antimalarial field was typical. The pantothenate-antagonists and 2-alkyl-3-hydroxynaphthoquinones (anti-vitamin K?[2]) were outclassed by compounds less recognizably antimetabolite in structure. These disappointments were eased somewhat by evidence that two major antimalarials, the chloroguanide metabolite (1) and its close relative pyrimethamine (2a) are potent inhibitors of dihydrofolate reductase.[3]



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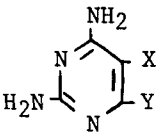


2

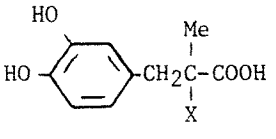
2a) X = 4-ClC$_6$H$_4$; Y = Et

2b) X = 3,4,5-(MeO)$_3$C$_6$H$_2$CH$_2$; Y = H

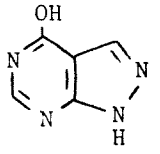
Adrien Albert's "Selective Toxicity"[4] provided a timely diagnosis of errors in early use of the antimetabolite drug strategy. Many apt illustrations of ways to achieve selectivity are drawn also from agricultural pest control, the other major area of applied comparative biochemistry.

In the interpretation of drug mechanism necessary for corroboration of theory, the whole picture develops slowly. Quite soon after Woods' discovery of the PABA-sulfa relationship, PABA was identified as a folic acid moiety[5] and the specificity of sulfas for dihydrofolate-synthesizing bacteria was demonstrated.[6] However, many years passed before dihydropteroate synthase was identified as the target enzyme.[7] Production of the key evidence awaited development of improved methods for enzyme separation; a nice turnabout is the recently-reported purification of dihydropteroate synthase by affinity chromatography on a sulfonamide linked to Sepharose.[8] Even now, gaps remain in sulfa biochemistry. There is a shortage of data on dihydrofolate synthase, the next enzyme in cofactor biosynthesis, and upon closer inspection, one finds many subtle, unsolved problems, particularly relating to resistance and synergism phenomena. Still the situation here is tidier than most.

Today, thirty-six years after Woods' seminal finding, there is abundant evidence for acceptance of the antimetabolite approach to drug design. It has been very effectively championed by leaders in medicinal chemistry, notably by B. R. Baker[9] and G. H. Hitchings.[10] The Hochster-Quastel text, "Metabolic Inhibitors"[11], which covers medicinal applications, now runs to four volumes. J. H. Quastel, incidentally, first demonstrated antimetabolite inhibition, with block of succinic dehydrogenase by malonate.[12] There are many successful antimetabolite drugs: the antihypertensive methyldopa (3a)[13], allopurinol (4)[14] for use in gout, the antibacterial trimethoprim (2b)[15], the coccidiostats amprolium (5a)[16a] and ethopabate (6)[16b], monoamine oxidase inhibitors used in mental health therapy, anticancer drugs and many others. The antiviral virazole (7)[17] is a new arrival. Activity continually extends to new areas; illustrative is a recent report on blockade of ovulation in rats by inhibitory analogs of the polypeptide luteinizing hormone-releasing hormone.[18] Perhaps most significant, since chemists are pragmatists, is the fact that approximately one-third of the articles which appeared in the Journal of Medicinal Chemistry in 1975 have clear antimetabolite orientations.



	X
3a)	NH_2
b)	$NHNH_2$



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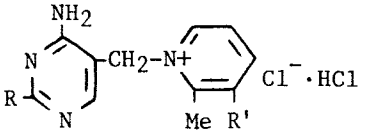
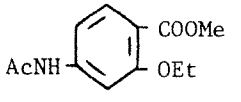
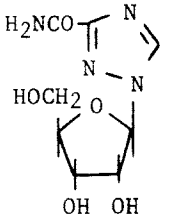


	R	R'
5a)	n-Pr	H
b)	Me	CH_2CH_2OH
c)	Me	$CH_2CH_2O\underline{PP}$



6



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Biochemical Strategies - The first step toward design is a decision on biochemical strategy. There are three alternatives: exploitation of a known drug mechanism, discovery of a strategy and conception of a strategy on theoretical grounds.

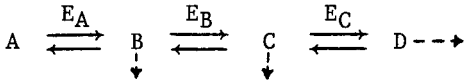
Exploitation of a known mechanism requires little comment here, since most of the reasons which may justify this route for a research team, such as development of an improved assay or new ideas in synthesis, are not germane. The same is true for modifications of a parent drug which affect distribution or metabolism. However, the inhibition of metabolizing enzymes is a valuable method which deserves mention. As an example, the DOPA-decarboxylase inhibitor, L-α-hydrazino-α-(3,4-dihydroxybenzyl)propionic acid (carbidopa) (3b),[19] is used in the drug Sinemet to slow destruction of L-DOPA so that its anti-Parkinsonism action is prolonged. We shall discuss later methods

for increasing the specificity of inhibitors, which may relate to interest in elimination of drug side effects. One final observation: the biochemistry of a drug class is by no means fixed; as the structure changes in successive products, significant alterations often occur in target enzymes or receptors.

Discovery of the mechanism of action of an interesting drug at the enzyme level may be very profitable since the reward is the first chance at rational exploitation. To be acceptable, a mechanism must make sense in terms of the relation of drug level to K_i for the target enzyme, the physiological response elicited and its similarity to responses observed with other inhibitors of the same enzyme. Positive reversal experiments are corroborative. For antimicrobials, there are additional criteria relating to enzyme level resistance.

The antihypertensive drug methyldopa (3a) provides an example of the difficulty in mechanism identification. The compound was designed to block norepinephrine synthesis at the DOPA decarboxylation step. Methyldopa does inhibit the decarboxylase, but its antihypertensive activity is now ascribed to the "false transmitter" activity of its metabolism product, α-methylnorepinephrine.[20] The decarboxylase inhibition mechanism was seen to be inadequate from the failure of other enzyme inhibitors to reduce blood pressure. For example, it was found that the hydrazino analog of methyldopa (3b) is a thousand-fold more potent enzyme inhibitor, yet lacks antihypertensive activity.

The methyldopa experience and other similar ones teach that biochemical strategies must be regarded as approximate only. In an enzyme catalyzed reaction sequence an inhibitor, designed to simulate C and thereby inhibit E_C, may actually inhibit E_B or E_A by product or feedback mechanism, or may compete with the substrates in associated metabolic or transport processes. Because of difficulty in pinpointing targets, test systems with three levels of complexity-pure enzyme, isolated target (e.g., plate assay) and intact animal-are minimal for satisfactory checks on design, strategy, delivery and host tolerance. There are many pitfalls in transferance from inhibition of pure enzyme to inhibition in cells and tissues.[21a]

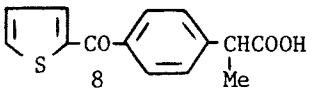


There is a second way to discover a strategy. Often the biochemistry of the system of interest is so incompletely understood that a suitable plan seems beyond reach. In this case screening with a random assortment of established antimetabolites may reveal a critical enzyme. As an example, O-methylthreonine, a recognized isoleucine antagonist, was found active in an avian mycoplasma assay, indicating that isoleucine is a critical requirement of the organism and a good subject for further inhibitor design efforts.[22] Presumably antimetabolite probes could locate sensitive areas even in complex physiological processes (e.g., fertilization), where the biochemistry would defy analysis. A well-stocked shelf of inhibitors is needed. When possible, it is desirable to employ several significantly different inhibitors of the same enzyme or analogs of the same metabolite.

De novo strategies must meet criteria for selective toxicity and practicality. Obviouly the best targets are peripheral, species-specific enzyme systems. Involvement with basic biochemistry increases the danger of host toxicity and, also, the probability of alternative pathways, hence resistance development or drug tolerance.

In practice medicinal chemists cannot wait for solid biochemical rationales. Many of their discoveries are "premature." Instead, medicinal chemistry often poses problems and provides tools for the biochemist. As an example, thiamine transport inhibition has been shown to be the mechanism of action of the coccidiostat amprolium (6a), but active transport of thiamine had not been considered seriously before the arrival of the drug.[23] Once given a lead, biochemical studies are helpful for its development. Gertrude Elion's summary of enzyme specificity studies on purine analogs[24] illustrates this point well.

A practical consideration in choice of target is the level of metabolite (M) with which the inhibitor (I) must compete. An inhibition index (the [I]/[M] ratio required to produce a specified effect[21b]) of 10 is rare; 100 is more likely. For this reason the synthesis and utilization of cofactors and hormones, which are present at ppm levels, are favored targets. Prostaglandin biosynthesis inhibitors, such as suprofen (8)[25] are now receiving much attention.



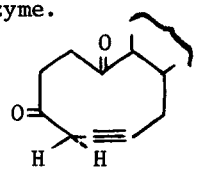
8

Transport proteins and mechanisms such as the controversial γ-glutamyl cycle for amino acid transport[26] may be vulnerable "bottlenecks." Because of the importance of aromatic and heteroaromatic amino acids in pharmacology, it is interesting to learn that L-phenylalaninol blocks phenylalanine uptake.[27] Perhaps the amino alcohol forms an N-γ-glutamyl derivative and the corresponding oxazoline.

Design - We shall emphasize those aspects of antimetabolite design most pertinent to medicinal chemistry. A good starting point is the distinction between "classical" and "non-classical" inhibitors.[9] The hazard for the classical inhibitor, which has minimal structural change from the model metabolite, is host incorporation. This occurs frequently, always raises difficult questions in toxicology, and has ominous implications when nucleic acid synthesis is involved. Another disadvantage of classical inhibitors is that approximate isosteric limitations are placed upon design, whereas with a non-classical inhibitor, the steric tolerance exploited permits useful properties to be built in, such as exoalkylation potential, improved delivery, or diminished metabolism.

Classical design is required for bioactivation, as when a purine analog must be converted to a ribotide, but bioactivation strategies of this sort are dubious, being highly susceptible to resistance development. Cohen[28] has suggested that the therapeutic value of nucleosides and nucleotides has been underestimated.

Transition state[29] and k_{cat}[30] inhibitors have been discussed in recent volumes of this series. They have great appeal to the bioorganic chemist. Long fascinated by enzyme catalysis, he now is presented with opportunities to exploit and thereby confirm hypotheses on mechanisms. Although these approaches have been well known for only a few years, a substantial number of novel and extremely effective enzyme inhibitors has been reported. A good example of "enzymic suicide" is the Δ^5-3-ketosteroid isomerase inactivation effected by 5,10-seco-19-norcholest-5-yne-3,10-dione (9)[31] Abstraction of the 4-β-hydrogen leads to an allenic structure which alkylates the enzyme.



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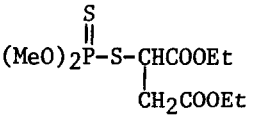

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Compounds of these types are not likely to be selectively toxic. Design is focused on the catalytic center, precisely where there is the greatest similarity, even identity, between the enzyme of different species. Hence an enzyme common to both host and pathogen is a poor target. Of course other avenues to selective toxicity are still open. An example is the insecticide-ectoparasiticide malathion (10), which is selectively activated by insects (P=S → P=O) and selectively inactivated by mammalian species (ester hydrolysis). Organophosphates are not generally recognized as transition state inhibitors, but long ago Orgel[32] called attention to the similarity between their structures and the transition state in ester hydrolysis.

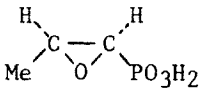
The potency of transition state and k_{cat} compounds may be critical for systems in which the substrate level is high. Transition state inhibitors have remarkably low K_i values, generally several orders of magnitude below the K_m for the enzyme reaction, yet, according to the theory advanced,[29] the K_i figures should be much lower, conservatively by a factor of 10^{-6}! A less demanding mechanism is suggested by Koshland's "induced fit" proposal.[33] If ground state and active enzyme forms are designated by E and E', the reactions of a transition state analog (TXA) may be represented as follows:

$$E + TXA \underset{\leftarrow}{\overset{\rightarrow}{}} E\cdot TXA \underset{\text{slow}}{\overset{\text{fast}}{\rightleftarrows}} E'\cdot TXA$$

Reversion of E'·TXA to E·TXA should be slow because the analog has been designed to fit the active enzyme form better. The equilibrium should be to the right and the concentration of free enzyme low.

Most medicinal chemists are familiar with Baker's concepts of enzymic inactivation with endo and exo alkylating and acylating agents.[9] To be useful as a drug, an antimetabolite of this type must react "intramolecularly," that is, only within the enzyme active center. This is a difficult design assignment, but its feasibility is demonstrated by the specificity of fosfomycin (11). This antibiotic reacts only with bacterial pyruvate-

uridine diphospho-N-acetylglucosamine transferase.[34] Specific endoalkylation occurs after enzyme activation of k_{cat} inhibitors. The previously-mentioned limitations of transition state and k_{cat} inhibitors with respect to selective toxicity apply also to *endo* alkylaters and acylaters, but not necessarily to *exo* agents.



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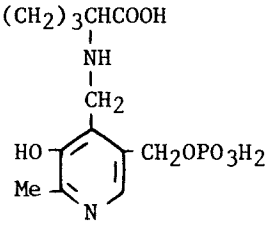

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In designing an antimetabolite, it is important to reduce or eliminate effects on off-target reactions. Several examples may be enlightening. Amprolium (6a), previously cited as a thiamine transport inhibitor, lacks the hydroxyethyl group present in the vitamin and its classical antagonist pyrithiamine (6b). Conversion to a pyrophosphate is impossible, hence the undesirable involvement in cocarboxylase (B_1-PP)-mediated reactions, which happens with pyrithiamine-pyrophosphate (6c), is avoided. We should note in passing that pyrithiamine, made by Elderfield and Tracy, was the first designed antimetabolite.[35]

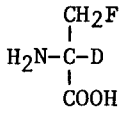
The vitamin B_6 antagonist, 4-deoxypyridoxine, is phosphorylated *in vivo* and is notoriously non-specific, blocking many pyridoxal and pyridoxamine phosphate-catalyzed reactions. Selectivity may be bred in by making an analog of a substrate-cofactor conjugate, such as N-(5-phosphopyridoxyl)-ornithine (12), a potent inhibitor of ornithine decarboxylase.[36]

As a final example, leupeptin trypsin inhibitors [acetyl(or propionyl)-Leu-Leu-argininal] are transformed into chymotrypsin inhibitors when the argininal moiety is replaced by phenylalaninol, tyrosinal or tryptophanol.[37]

Because of space limitations, this review cannot do justice to the countless ingenious structural modifications which chemists have employed in antimetabolite design. Personal favorites are Belleau's use of tropolones to inhibit catechol O-methyltransferase,[38] the substitution of a carboxymethyl for a phosphate group, suggested by PEP and itaconate inhibition of isocitrase[39] and the "quaternary equivalence" of hindered amines observed with ganglionic blockers such as 1,2,2,6,6-pentamethylpiperidine (pempidine).[40] 2-Deutero-3-fluoro-D-alanine (13) is a splendid example of design. This is the first use of deuterium in a drug. The D-alanine antagonist, when administered with a stabilized cycloserine derivative which possesses specific pharmacokinetic properties, shows very promising antibacterial action.[41]

Every device employed in design seems to have been anticipated by microorganisms. Probably we have not caught up yet. The enzyme inhibitors from fermentation broths, which are now being studied by Umezawa and others,

have most surprising structures. Typical are the dopamine β-hydroxylase inhibitors, fusaric acid[42] and phenopicolinic acid[43] or, respectively, 5-n-butyl and 5-(4-hydroxybenzyl)-picolinic acids; a glyoxalase inhibitor, 2-crotonyloxymethyl-4,5,6-trihydroxycyclohex-2-enone;[44] an N-methyltransferase inhibitor, 1-(2,3,5,6-tetrahydropyridyl)-1,3-pentadiene;[45] pyrindolol (14), a β-galactosidase inhibitor;[46] and the steroid hydroxylase inhibitor diplodialide A (15).[47] These are undoubtedly only the first tappings of a new lode. Earlier work on antimetabolites from microorganisms was reviewed recently.[48]



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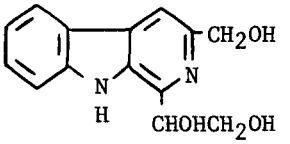

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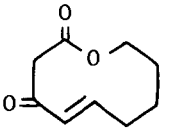

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Actually many enzyme and receptor inactivators lack credible resemblance to the normal substrates. Other mechanisms may be involved, such as disruption of enzyme ternary structure. To be effective in this way, an agent would have to possess groups, probably two or more, which are capable of binding competitive with internal enzyme forces and are placed so as to pair with a template constellation in the enzyme. The phenomenon of binding and inactivation by non-substrate ligands is recognized, but our present ignorance of ternary enzyme structures precludes detailed explanations.

At some future date, the design of effective enzyme inhibitors should become an exercise in solid geometry, followed by challenging synthetic chemistry. Given the exact dimensions of an enzyme, as it exists in solution or in its functional state, perhaps immobilized in a membrane or multienzyme complex, the chemist should be able to identify appropriate binding groups in the active center region, so that a molecule may be designed to pair with these groups, prevent access of the usual substrate or cofactor and block enzyme operation. For a selectively toxic enzyme inhibitor, with useful drug potential, there would be superimposed the requirement that the binding sites selected differ in some utilizable way from sites in similar host enzymes. This is what has in effect been achieved, by a trial and error process, with the bacterial dihydrofolate reductase-specific inhibitor, trimethoprim (2b). The most incisive proof of the possibility for drugs with species specificity is data on the K_i values of pyrimethamine and trimethoprim for bacterial, protozoal and mammalian reductases.[15]

The beautiful X-ray studies of lysozyme by Phillips[49] and similar research on esterases and amidases by Kraut[50] and others offer the medicinal chemist a vision of future possibilities for precise design. Quicker, cheaper approaches to protein crystal structures have been suggested.[51] Moreover, methods for prediction of protein folding may reduce dependence on X-ray crystallography.[52] Improvements in technique are needed to solve such formidable problems as the structure of leukemic cell dihydrofolate reductase.

All things considered, the reviewer endorses George Hitching's sug-

gestion that "enlightened empiricism" is the best philosophy for medicinal chemists.

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Chapter 25. Comparative Toxicology

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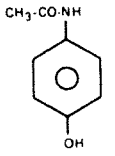
Many foreign compounds are converted in the body to chemically reactive metabolites that react with various cellular components including small molecular weight substances such as lipids, glutathione and water with macromolecular substances including proteins, glycogen, DNA and RNA or with oxygen to produce superoxide or hydrogen peroxide. Occasionally these reactive metabolites cause various kinds of toxic effects including, cancer[1-4], mutagenesis[5], cellular necrosis[6,7], hypersensitivity reactions, fetotoxicities, methemoglobinemia and blood dyscrasias[7]. However, the mechanisms by which the metabolites evoke their toxic effects are frequently obscure. Reactive metabolites of carcinogens and mutagens are thought to evoke their toxicities by reacting with DNA. In another example, trichloromethyl free radical, the reactive metabolite of carbon tetrachloride and trichloromonobromomethane, is thought to cause liver necrosis by promoting lipid peroxidation[6]. However, lipid peroxidation cannot be the only mechanism of liver necrosis because bromobenzene, acetaminophen (I) and furosemide (II) do not cause lipid peroxidation in liver even though they cause liver necrosis. The destruction of adrenergic neurons by 6-hydroxydopamine (III) is thought to be mediated either by the covalent binding of the quinone (IV) or by the superoxide formed during the autoxidation of the drug within neurons[9].



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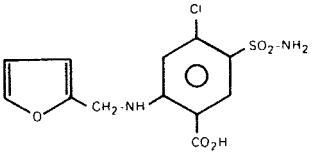

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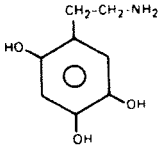

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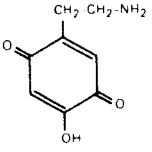


IV

The usual approach to studies in drug metabolism cannot be applied to studies of reactive metabolites. When a metabolite is highly reactive, the chances of isolating enough of it from tissues to identify are slim and the chances of developing an accurate quantitative method are even less. Moreover, even if we were able to develop methods, the measurement of the concentration of the reactive metabolite at any given time provides only part of the information required to estimate the total exposure of the tissue to the metabolite. Furthermore, even when we know the identity of the reactive metabolite and are able to synthesize it, there is no assurance that

the reactive metabolite would reach the target organ after its administration either orally or parenterally. For this reason indirect methods have been used to estimate the formation of reactive metabolites.

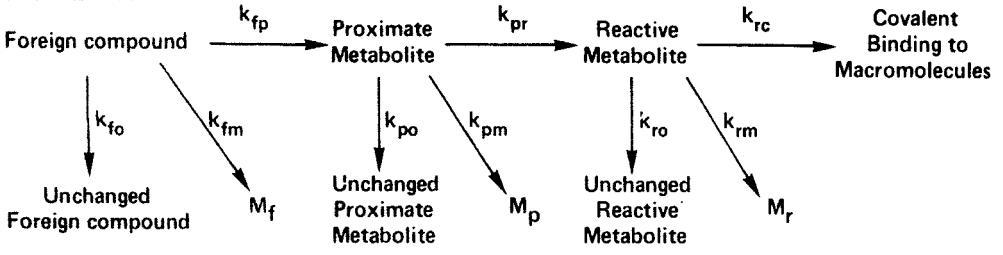
Advantage has been taken of the fact that very chemically reactive metabolites react rather indiscriminately with various tissue macromolecules including proteins, the various RNA's, DNA, glycogen and lipids even though the relative rates at which these reactions take place may vary widely with different reactive metabolites. Most reactive metabolites including those that are carcinogenic and those that evoke lipid peroxidation react with protein and hence some investigators have used the measurement of covalent binding of reactive metabolites to proteins as an indirect measure of the exposure of the tissue to the reactive metabolite and the chemical reactivity of the metabolite. When it is suspected that the reactive metabolite exerts its toxicity by reacting with other kinds of macromolecules, the covalent binding of reactive metabolites to these macromolecules are also measured. It is especially noteworthy that measurements of the covalent binding of reactive metabolites to macromolecules are useful even when the mechanism by which the reactive metabolite causes toxic effects does not cause covalent binding of the metabolite to tissue macromolecules. For example, the trichloromethyl free radical formed from carbon tetrachloride is thought to promote lipid peroxidation in liver by reacting with unsaturated fatty acids in phospholipid to form chloroform and fatty acid free radicals that react in turn with oxygen to form lipid peroxides[6]. Thus, according to this mechanism covalent binding of trichlororethyl free radical to lipid is not involved in the initiation of lipid peroxidation. Nevertheless, some of the trichloromethyl free radical does combine covalently with phospholipid and thus changes in the rate of formation of the trichloromethyl free radical should change the rates of both reactions. Therefore, changes in the rate of covalent binding should approximately parallel changes in the rate of formation of the fatty acid free radical and hence lipid peroxidation and liver necrosis.

The amount of reactive metabolite covalently bound to liver protein *in vivo* may be viewed as the mathematical product of the dose of the toxicant times a series of ratios[10]. The length of the series depends on the number of sequential reactions by which the foreign compound is converted to its chemically reactive metabolite. For example, suppose that a chemically inert foreign compound is converted to a chemically inert intermediate that in turn is converted to its chemically reactive metabolite that becomes covalently bound to various macromolecules. In this situation, the equation for the fraction of the dose that becomes covalently bound is as follows:

$$F = A\ B\ C \qquad (1)$$

In which (A) is the proportion of the foreign compound that is converted to the chemically inert intermediate (B) is the proportion of the chemically inert intermediate that becomes converted to its chemically reactive metabolite, and (C) is the proportion of the chemically reactive metabolite that becomes covalently bound to tissue macromolecules.

Changes in the amount of metabolite covalently bound to tissue macromolecules after a given dose of the toxicant can occur only by changing one or more of the ratios, A, B, etc. However, each ratio depends not only on the rate at which the toxicant or metabolite is converted to the next compound along the pathway leading to the covalent binding but also on the rate at which it is eliminated from the body along innoxious pathways. When one or more of the pathways are catalyzed by enzymes or transport systems that become saturated by the toxicant or metabolite or when one or more of the pathways are catalyzed by enzyme systems that require cosubstrates which may become depleted in the tissue, the relative rates will vary with time and therefore the ratios are difficult to visualize. In order to gain an insight into the interrelationships among the various pathways, however, let us assume that all processes are first order, that is the rates are directly proportional to the concentration of the parent compound or the metabolite. Under these conditions, each ratio equals the rate constant for the formation of the next metabolite on the road to the reactive metabolite divided by the sum of the rate constants for all of the reactions by which the compound or metabolite is eliminated from the body (Fig. 1).



$$A = \frac{k_{fp}}{k_{fp} + k_{fo} + k_{fm}} \qquad B = \frac{k_{pr}}{k_{pr} + k_{po} + k_{pm}}$$

$$C = \frac{k_{rc}}{k_{rc} + k_{ro} + k_{rm}}$$

Covalent Binding/Dose = F = ABC

Fig. 1. Pathway for the formation of a hypothetical chemically reactive metabolite.

Obviously each ratio can be changed by altering either its numerator or denominator. Thus, the greatest change will occur when the numerator is altered and the denominator either remains virtually unchanged or is altered in the opposite direction. This will occur when the rate constant for the formation of the metabolite leading to the toxic metabolite is small compared with the sum of the rate constants for the elimination of the drug or its inert metabolite and when the treatment either alters only the reaction leading to the toxic metabolite or has opposite effects on this reaction and the major reaction by which the substance or the inert

metabolite is eliminated. On the other hand, the ratio may be changed very little when the reaction leading to the formation of the reactive metabolite is the major mechanism of elimination or when all of the reactions are changed to about the same extent. Thus, treatments that alter the rates of metabolism of the foreign compounds and their metabolites will alter covalent binding and the toxicity of chemically reactive metabolites only when such changes result in changes in one or more of the ratios. Moreover, a given treatment may increase one ratio but decrease another and thus the change in the covalent binding and toxicity may not be as great as might be expected from measurements of only one of the ratios.

The denominator of A may be calculated from the biological half-life of the parent compound, whereas changes in the numerator of A may be frequently detected by enzymatic studies of covalent binding of the parent drug *in vitro*. Moreover, the relationship between the urinary metabolites and the various ratios becomes obvious when it is realized that (1-A) equals the proportion of the dose that is eliminated from the body by pathways other than that which leads to the formation of the chemically reactive metabolite; (1-B) is the proportion of the proximate metabolite that is eliminated by pathways other than that leading to the formation of the chemically reactive metabolite; etc. Thus, the ratio concept provides a means of evaluating the interrelationships not only between *in vivo* covalent binding of the reactive metabolite and toxicity but also between the *in vivo* covalent binding and the pattern of drug metabolites in urine, breath and feces, the biological half-life of the parent compound in the body and the *in vitro* binding by various tissue preparations.

This integrated approach has been useful in elucidating the reasons for many of the species differences in the toxicity of many foreign compounds mediated by chemically reactive metabolites, including the commonly used analgesic, acetaminophen.

Large overdoses of acetaminophen cause fatal hepatic necrosis not only in man[11] but also in several laboratory animal species including rats, mice and hamsters[12]. However, there is a marked difference in the sensitivity of the various species to the drug (Table 1). In hamsters, necrosis occurs in most of the animals even at doses as low as 150 mg/kg, whereas in rats, necrosis occurs in less than 10% of the animals even at doses as high as 1.5 grams/kg[12]. Studies during the past few years have shown that the liver necrosis found after high doses of acetaminophen is caused by a chemically reactive metabolite and that the marked species differences in the incidence of liver necrosis is due to species differences in the formation and inactivation of the metabolite. But without studies on the covalent binding of the reactive metabolite to liver proteins it seems unlikely that the reason for the species difference would have been discovered.

Acetaminophen administered to animals is excreted mainly as its glucuronide and its sulfate conjugates but a small amount of the drug is excreted as its mercapturic acid and cysteine derivatives in all animals

studied including man. Hence the patterns of urinary metabolites of acetaminophen after large doses are administered surprisingly similar in different animal species. Even, the biological half-life of acetaminophen in rats is surprisingly similar to that in mice (Table 2)[14].

Studies on the covalent binding of radiolabel to liver protein after the administration of various doses of radiolabeled acetaminophen to mice revealed that only negligible amounts of the drug were covalently bound at doses below 100 mg/kg (Table 3)[15]. At higher doses, however, considerable radiolabel was covalently bound to liver protein. Moreover, the covalent binding appeared to be negligible until the liver was depleted of glutathione. Since acetaminophen is chemically inert, these findings thus indicated that it was converted to a chemically reactive metabolite in mice. They further suggested that at therapeutic doses of the drug, virtually all of the reactive metabolite is converted to a glutathione conjugate that is ultimately excreted as a mercapturic acid. But at toxic doses of the drug, the glutathione in liver is decreased to such an extent that the reactive metabolite can no longer be completely inactivated by glutathione and thus a portion of it becomes covalently bound to liver proteins. In accord with this view, the proportion of the dose of acetaminophen that is excreted as the mercapturic acid is about 10% when nontoxic doses of the drug is administered to mice and decreases as the dose is increased[13]. On the other hand, a dose of 300 mg/kg administered to rats does not significantly decrease the glutathione in liver[12]. Moreover, the proportion of the dose that is excreted as the mercapturic acid is only about 4% when low doses are given to rats and does not significantly change when the dose is increased[13].

Studies with liver microsomes indicated that the formation of the chemically reactive metabolite, as measured by covalent binding of radiolabeled acetaminophen to microsomal protein, is catalyzed by a cytochrome P-450 enzyme in liver microsomes. They also showed species differences in the kinetics for the formation of the reactive metabolite[12]; with liver microsomes from mice, the apparent maximal velocity for the reaction (V_{max}) was 0.18 nmoles bound/mg protein/min and the apparent K_m was about 0.36 mM, whereas with liver microsomes from rats, the apparent V_{max} was .07 nmol/mg protein/min and the apparent K_m was 14.8 mM. Since the intrinsic clearance of a substrate by an enzyme in the body should be $V_{max}/(K_m + S)$, these findings are in accord with the view that the rate of formation of the reactive metabolite would be slower in rats than in mice not only because the V_{max} is lower in rats than in mice, but also because the K_m is higher.

Since the reactive metabolite preferentially combines with glutathione and other thio-nucleophiles, such as cysteine and cysteamine, the depletion of liver glutathione by other substances that react with glutathione should increase the covalent binding of the reactive metabolite, whereas the administration of cysteine and cysteamine should decrease it. In accord with this view, diethylmaleate, which decreases the concentration of glutathione in liver but does not cause liver necrosis, increases not only the covalent binding of the reactive metabolite of acetaminophen by increasing ratio B but also increases the incidence and severity of the

liver necrosis in mice[15,16]. On the other hand, treatmentof mice with cysteine decreases the covalent binding of the reactive metabolite by decreasing ratio B and decreases the incidence and severity of the liver necrosis[15].

Table 1

Liver necrosis caused by acetaminophen

Dose (mg/kg)	Incidence (%)		
	Mice	Hamsters	Rats
150	0	0	-
200	-	20	-
300	22	89	-
375	46	-	-
425	-	100	-
500	76	-	0
750	99	-	-
1000	-	-	2
1500	-	-	6

Data taken from Mitchell et al.[14]; Potter et al.[16]

Table 2

Species differences in acetaminophen metabolism

	Mouse (400 mg/kg)	Rat (400 mg/kg)
	% of dose	
Acetaminophen	14.1	18.0
glucuronide	60.7	34.2
sulfate	19.5	41.0
mercapturic acid	6.5	3.8
$t_{1/2}$ of acetaminophen		
mouse (375 mg/kg)	27 min	
rat (350 mg/kg)	66 min	

Data taken from Jollow et al.[13] and Mitchell et al.[14]

Table 3

Covalent binding of the reactive metabolie of acetaminophen to mouse liver protein

Dose mg/kg	Covalent binding nmoles/mg protein
5	< 0.10
25	< 0.10
100	< 0.10
400	0.70
800	1.75

Data taken from Mitchell et at.[15]

Pretreatment of mice with phenobarbital increases the activity of the cytochrome P-450 enzyme in liver microsomes that catalyzes the formation of the reactive metabolite[17], but apparently does not affect the enzymes that catalyze the formation of the sulfate or the glucuronide conjugates because it does not alter the biological half-life of the drug[14]. Thus, pretreatment of mice with phenobarbital increases the proportion of the dose of acetaminophen that becomes covalently bound to liver protein by increasing ratio A and increases the incidence and severity of the liver necrosis.

Nevertheless, there is a species difference in the effects of various treatments. For example, the pretreatment of hamsters with phenobarbital only slightly increases the formation of the reactive metabolite of acetaminophen[16] but markedly increases the rate of formation of the glucuronide conjugate[13] and therefore decreases the biological half-life of the drug[16]. Thus, the pretreatment of hamsters with phenobarbital decreases the proportion of high doses of acetaminophen that becomes covalently bound to liver protein by decreasing ratio A and decreases the incidence and severity of the liver necrosis[16]. The effect of phenobarbital pretreatment on the toxic actions of acetaminophen thus depends on its relative effects on all the enzymes required for the metabolism of the drug *in vivo* and cannot be predicted by studying its effects on only one of them, as frequently occurs in studies *in vitro*.

Summary - It became obvious many years ago that species differences in the metabolism of reversibly acting drugs could markedly affect the pharmacological and toxicological effects of the drugs[18]. But in recent years it has become evident that studies on the pattern of urimary metabolites and the biological half-lives of drugs and their primary chemically inert metabolites fail to reveal relevant species differences in the metabolism of chemically reactive metabolites that mediate toxic effects. Combined with studies on the covalent binding of the reactive metabolites both *in vivo* and *in vitro*, however, such studies aid in clarifying the reasons

for many of the species differences in drug toxicity mediated by reactive metabolites. It has become evident that there are species differences not only in the absolute rates of various drug metabolizing enzymes but also the relative activities of these enzymes. Indeed, differences in their relative activities are frequently more relevant in accounting for species differences in the toxicity of reactive metabolites than are differences in their absolute rates. Indeed, this will be true whenever the rate constants for the formation of the reactive metabolite are much greater than the rate constant for the repair or replacement of the altered substances in target tissues. Moreover, there are species and strain differences in the effects of various substances that increase and decrease the activities of the various drug metabolizing enzymes. Some treatments may alter the activities of several different kinds of enzymes, but their relative effects on these enzymes may differ markedly from one animal species to another and perhaps from one strain to another within a given animal species. There are also species differences in the substrate specificity of the enzymes affected by various inducers. For example, the form of cytochrome P-450 induced by 3,4-benzpyrene in rabbits differs markedly from the form induced in rats[19]. How such differences as these will affect the metabolism of a given compound in a given strain of animals is difficult if not impossible to predict with accuracy and thus data showing species difference in drug metabolism and toxicity should be extrapolated to different compounds or different strains of animal species with caution.

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Chapter 26. Chronopharmacology - Its Implication for Clinical Medicine

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Introduction: In recent years, biologists have become increasingly aware of the dimension of time. Today chronobiology is a well-established field; and one of its subdisciplines, chronopharmacology, is becoming particularly important to the practicing physician.

This paper will deal first with some generalizations about mammalian chronobiology, then introduce the reader to several descriptive terms and cite examples of biological rhythms. Later, attention will be focused on chronopharmacological implications.

Generalizations: (1) Oscillation is a fundamental property of all animal and plant life; it also characterizes all levels of organization from the molecular to that of the whole organism.

(2) The frequency spectrum of rhythms is broad, ranging from fractions of a minute to days or months or even a year. This paper will focus on the circadian rhythms, or those with a frequency of about a day. It should be kept in mind that rhythms of higher (ultradian) or lower (infradian) frequency may be superimposed on the circadian frequency.[1]

(3) Initially many thought that circadian rhythms were merely a reflection of some rhythmic geophysical event such as the rising or setting of the sun or perhaps just a simple response to the ingestion of a meal. On the contrary, such rhythms are endogenous and innate. They are, however, capable of being synchronized to an external force. In the case of animals it is generally accepted that the light-dark cycle of nature or that of the laboratory is the predominant synchronizing force; in the case of man it is the social cycle. By synchronization it is implied that the endogenous frequency is capable of being coupled and driven by an exogenous synchronizing cycle (Zeitgeber is the German equivalent).

(4) Endogenous rhythms continue to oscillate in the absence of a dominant synchronizing force providing no secondary synchronizers are present. Under such circumstances, these rhythms fluctuate with a frequency that no longer is precisely 24 hours but only approximates this frequency. Consequently, if one monitors a particular phase (such as the trough or peak) of the freerunning rhythm from day to day, it will continually change in relation to local clock time. The behavior of freerunning rhythms in man currently is under intensive investigation, and it is essential that one understands the concept of freerunning and the many factors influencing it.[2]

(5) On a day-to-day basis the circadian system serves to adjust the organism to changing events such as the sleep-wake cycle. For example, it has been reported repeatedly that cortisol levels in the blood begin

increasing in man prior to awakening - presumably to prepare him to adjust to the more demanding events associated with the awakened state. It makes little difference if he remains up and active on a particular night; the steroid pattern of fluctuation will remain essentially the same as if he had slept.

(6) The ability of a rhythm to freerun or to be synchronized to a precise 24-hour geophysical cycle (or social cycle in the case of man) is the very mechanism that permits the organism to adjust in an orderly manner to a changing environmental cycle such as occurs with the changing seasons. Unfortunately it is this same mechanism that an organism must depend upon to adjust it to more rapid changes such as those experienced by the shift worker or by the traveler displaced through several time zones. While adjusting to such situations, different rhythmic variables shift relatively slowly and at different rates; this can cause temporary internal desynchronization among these variables - hence the cause of the discomfort commonly referred to as "jet lag".

(7) It has been documented that circadian rhythms are not necessarily dependent on food intake because for man certain rhythms (steroids, catecholamines, etc.) continue to oscillate in the fasting state.[4] Of course some variables such as serum glucose, etc., are strongly influenced by food intake;[4] and under certain circumstances, food intake can be made to override the strong synchronizing force of the light-dark cycle in animals[5,6,7] and of the social cycle in man.[8] This can be done by restricting food intake to a precise span of the day (for example, to four hours for rodents). Several rhythmic variables can be synchronized in this way; others show evidence of being synchronized to both restricted feeding schedules and to the light-dark cycle - the end result being a rhythmic waveform demonstrating an interaction between the two.[5,7] Interestingly, other variables remain strongly synchronized to the light-dark cycle.[7,9] We have elaborated somewhat on the role of food because there has been a surge of interest in the possibility of synchronizing rhythms to some easily manipulable factor or pharmacological agent which will dominate the synchronizing effect of the social routine or light-dark cycle. The objective is to control rhythms in healthy tissue in such a manner as to improve the therapeutic index in cancer chemotherapy. Further comment on this will follow.

Examples of Rhythms That Have Clinical Interest: Figure 1 illustrates in graphic form the average fluctuation seen along a 72-hour time scale for a number of diverse variables in a group of presumably healthy young men; the rest-activity schedules (shaded area) and meal times and menus were uniform for the group, but the quantity of food was not controlled.[4] The peaks for the memory and performance tests represent the times of poorest performance. Figure 2 is an acrophase map of 41 different variables in another similar group.[10,11] The data obtained were fitted to a 24-hour cosine curve by the method of least squares, and the rhythmic parameters were estimated (readily by a computer). The rhythmic parameters include the mesor (over-all 24-hour mean if data are equidistant), amplitude and acrophase. Only the acrophase is shown (point estimate, illustrated by a dot,

and 95% confidence intervals, horizontal bars); it represents the time when the crest of the rhythm occurs in relation to the rest-activity cycle. This is one of several methods currently used for quantifying time-series data.[12] The center column in Fig. 2 gives the average 24-hour range of change for the group - that is the percent difference between the lowest and highest recorded means (temperature change is in °F).

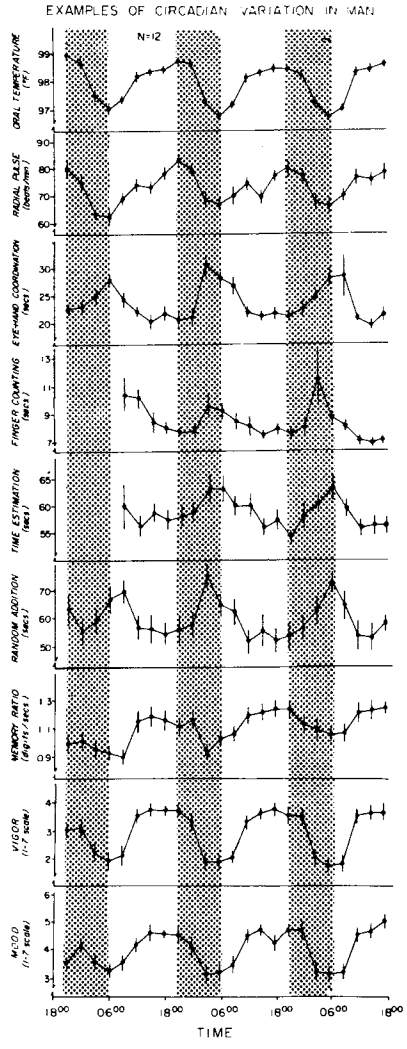

Fig. 1 - Plot of mean values and SE for the group over a 72-hour span with sampling at 8-hour intervals. (From Ref. 4 with approval of publisher.)

Averaging data may be misleading simply because any of the estimated parameters may vary greatly in the individual; this is especially true for the amplitude and mesor. For example, the range of change in oral temperature over the 24-hour span was about 2°F. For pulse, the range of change averaged 30% for the group, but the change was as great as 81% and as small as 21% for the single individual. The range of change for

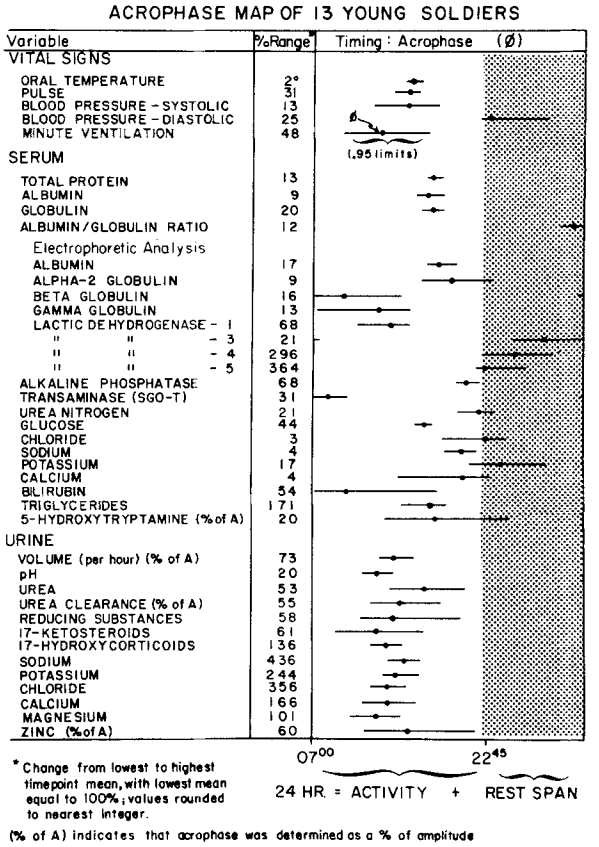

Fig. 2 - Acrophase map (see text). (From Ref. 10 with approval of publisher.)

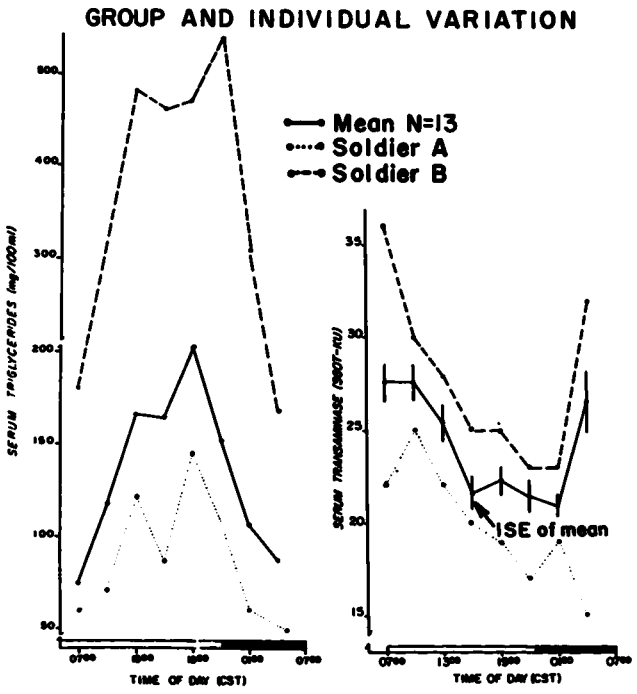

Fig. 3 - Individual and group variation in serum triglycerides and transaminase. (From Ref. 10 with approval of publisher.)

systolic blood pressure was 15% for the group, but for the single individual it was as great as 57% and as small as 10%. Diastolic blood pressure changes for the group averaged 14%, but were as great as 78% for a single individual and as small as 13% for another individual. At one phase of the circadian cycle, two of the 13 men had blood-pressure readings that might suggest at least diastolic elevation (150/108 and 148/96); the lowest readings recorded for these men were 122/68 and 120/70.[10] One could cite similar deviations for each of the 41 variables listed in Fig. 2. Figure 3 is a graphic illustration of the deviations for two variables.[10] The individual and group variation for serum triglycerides and transaminase are shown for subjects consuming the identical diet.

It is noteworthy that a National Center for Health Statistics survey[13] reports that on the average human blood pressure varies during the day by no more than 3 to 4 mm Hg. Although undoubtedly the statement is true in averaged data, the variation for individuals is artificially reduced. Such statistics can be very misleading, especially when it can be demonstrated that the diastolic blood pressure in a presumably healthy resting subject can vary as much as ten times the amount mentioned in the survey report.[10,14]

From our own studies on several populations, including the elderly, and from studies of others, it seems evident that individuals fall into three categories: (1) those having a high range of change for a particular variable; (2) those having a low range of change; and (3) those (possibly the largest group) who fall in between these extremes. What, if anything, do the differences among the three categories imply as far as health is concerned? It is conceivable that the amplitude of a rhythm may be important in evaluating the physiology of man. Some variables in presumably healthy individuals do indeed approach so-called "abnormal" values at one time point in the circadian cycle; whereas at other times they are well within

conventional "normal" ranges. The question may be asked: Will the younger individual who shows high blood pressure readings (near the currently postulated limits of hypertension) only during the acrophase become a hypertensive at all phases of his circadian system with increasing age? Or are the different readings all within his normal range: Will a man with high intraocular pressure at one phase of his circadian system become a glaucoma victim?[10] How does the physiology of cholesterol metabolism differ between the man who shows a fluctuation of only 6% daily and another whose cholesterol level fluctuates as much as 110%?[10] Similar questions may be asked for almost any variable measured.

Chronopharmacological Implications: Since the biological system is rhythmically changing, it follows that the organism is biochemically a different entity at different circadian phases; therefore it reacts differently to an identical stimulus at different times.

Among the many categories of agents reported to elicit differing responses when given at different circadian phases are: (1) carcinogens, (2) tumor cells, (3) viruses, (4) bacteria, (5) antigens, such as sheep red blood cells, (6) physical agents such as X-rays and noise, and (7) a host of poisons, chemicals and drugs. The end points measured also have been diverse and have included: (1) mortality rate, (2) duration of sleep subsequent to a fixed dose of an anesthetic agent, (3) duration of time required for the onset of tremors subsequent to giving a drug capable of inducing them and (4) survival time and "cure" rate subsequent to treating L1210 leukemic mice on a therapeutic protocol designed to exploit the circadian system. We and others have recently reviewed this general phenomenon in some detail.[3,15,16] Three examples of such a response are illustrated in Fig. 4. It is evident that these responses vary dramatically. For example, the mean duration of sleep resulting from identical doses of pentobarbital sodium given to different groups of rats at different times during the rat's circadian cycle averaged 91 min. when the dose was given at one phase of the cycle and only 53 min. when given at another time. This same figure shows that whether or not an animal survives a potentially lethal, fixed dose of amphetamine may be circadian-phase dependent; for at one phase 76.6% died and at another time only 6.6%. The third example demon-

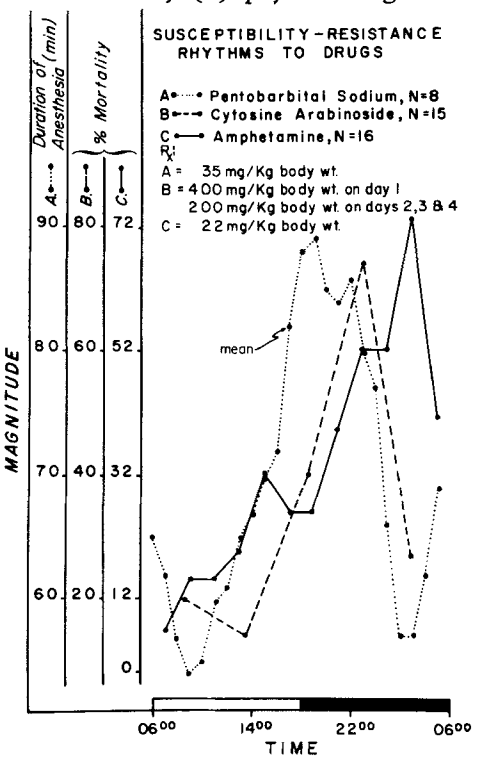

Fig. 4 - Circadian variation in susceptibility of rodents to 3 drugs. For more details on ara-C study, see Ref. 17.

strates that a carcinostatic drug, cytosine arabinoside (ara-C), is far more toxic at one phase of the mouse circadian system than at others.[17] Most of the other agents mentioned above are capable of eliciting equally dramatic circadian variation in response. Clearly the pharmacological response is circadian-phase dependent. Evidence now exists which challenges any pharmacokinetic study done without consideration of circadian variation. For example, our ethanolemia studies suggest that pharmacokinetic rates and rate-constants cannot be considered a priori to be unchanging throughout the 24-hour time span.[18] The possibility obviously should be investigated if pharmacokinetic results are intended to be used in calculating therapeutic doses of a drug. It may be inappropriate to administer a drug repeatedly over the 24-hour period on the basis of a single pharmacokinetic study performed at only one time of day.

Obviously the data mentioned thus far would suggest that there may be an optimum time for therapy. Halberg, a leading pioneer and major contributor in this field, has written extensively on the urgency of exploring timed treatment according to body rhythms.[19,20,21] To date, little has been done on a wide scale. Among those drugs whose effectiveness has been shown to depend on the phase of the circadian system when administered are antihistamines[22] and corticosteroids.[3,23] For example, Moore Ede[24] using oxymethalone reported that he could better manage calcium loss in his paraplegic patients by timing the administration of the drug. In this case the drug was dramatically more effective if given just before the peak in calcium urinary excretion; this was, as might have been predicted from Fig. 2, during the early morning hours. Most clinicians accept the idea that a firm understanding of the basic circadian rhythmicity of adrenal physiology is essential to routine steroid therapy, and many symposia have been held and papers reported on this subject.[3,23] Admittedly, significant advantages have been gained, but there still exists a tremendous potential for realizing even greater benefits of timed steroid therapy. To achieve this goal, one must recognize the importance of understanding the phasing of each individual patient's steroid rhythm, or indeed if his steroids are fluctuating in a rhythmic manner.[3]

We shall conclude by considering the potential for chronotherapy of cancer. As illustrated in Fig. 4, mice respond dramatically differently to cytosine arabinoside (ara-C)[17] given at different circadian phases. The question we asked was whether such information could be exploited when treating leukemic mice. Skipper et al.[25] showed that they were able to eradicate 10^5 leukemic cells, without animal deaths from drug toxicity, by giving eight treatments of 120 mg of ara-C per kg body weight divided into equal doses spaced at 3-hour intervals over a 24-hour span. Four such courses were given at 4-day intervals. Courses of 240 mg/kg of ara-C given on the same schedule were so toxic that they killed all the animals. With the latter data and with the chronobiologically determined host toxicity rhythm as background, an experiment was designed by Haus et al.[26] to determine if the tolerance of BDF_1 mice to 240 mg/kg courses of ara-C could be improved by applying chronobiological methods and concepts. This was done by inoculating BDF_1 mice with 10^6 L1210 leukemia cells and administering the 240 mg/kg of the ara-C in a special manner. Instead of giving the same

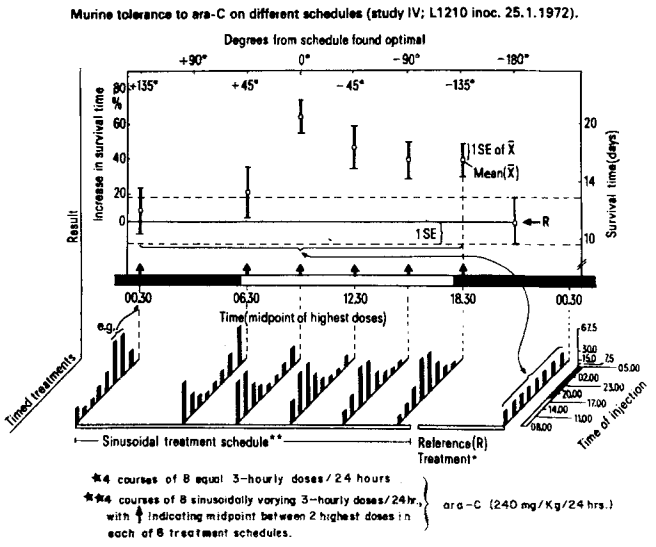

Fig. 5 - Comparison of sinusoidal and conventional treatment schedules. (From Ref. 26 with approval of publisher.)

amount of drug in eight equal doses at 3-hour intervals, as described above, it was given in a sinusoidally increasing and decreasing amount over the 24-hour span. The largest amounts were given at the time previously determined to be the time of peak host resistance to the drug, and the smallest doses were administered when the animals were most susceptible (Fig. 5). The application of this technique resulted in a statistically significant increase in survival time when compared to the reference schedule of equal doses. Hence it was reported that a carcinostatic drug, extensively used by clinicians, is tolerated better when administered according to the host's circadian rhythm in susceptibility than when administered on a conventional schedule designed without any chronobiological consideration.

Since that study, many others have been performed. Only recently it was reported that not only survival time but also "cure rate" in the mouse leukemic system can be improved.[27] Currently limited timed treatment schedules using combination drugs in cancer chemotherapy are being explored in the rodent. Remarkable reproducibility of data between different laboratories has been reported.[28] Certainly there is a right time to give a drug, as Halberg has advocated; but he also points out that there is a wrong time. Intensive studies are needed to come to a better understanding of timed treatment,[21] but there simply are not enough investigators working on this important problem.

We and others have been exploring rhythms in various organs in both the tumor-bearing and normal animal. High-amplitude circadian rhythms have been reported for the DNA synthesis in liver, bone marrow, gut, thymus and spleen of normal rodents; the presence of a tumor may dramatically alter the rhythm.[29,30,31] Such information is essential to designing better chemotherapeutic protocols. Limited studies will be undertaken in the near future to apply some of this information to the treatment of cancer in humans. The task will not be easy because of the logistical problems, but the benefits may make such an endeavor very worthwhile.

Earlier we mentioned that by restricting the intake of food to precise spans of time each day, one could cause certain rhythmic variables to be-

come syncrhonized to meal timing. Only recently it has been found that cell division in bone marrow, gut and spleen can be so synchronized.[32] Such data permit one to speculate that meal timing could be used to advantage in synchronizing these important rhythms in the patient undergoing chemotherapy.

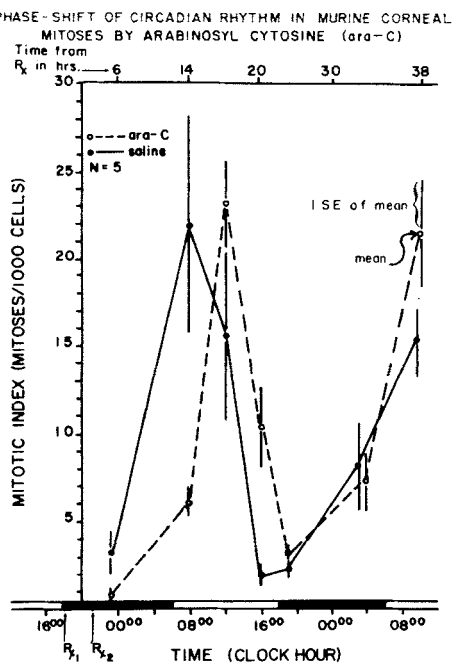

Fig. 6 - Murine tolerance to ara-C on different treatment schedules. For more detail, see Ref. 34. (From Ref. 34 with approval of publisher.

Certain drugs have been shown to phase shift rhythms.[33,34,35] The degree and direction of phase shift depends on when the drug is given. For example, if ara-C is given at 0900 (when the mitotic index is high), no effect is seen on the subsequent mitotic cycle.[3] If, however, the drug is given at the time of minimum mitotic activity, the next day the expected peak at 0900 is delayed for several hours (Fig 6).[34] Recently it was demonstrated that the rhythm in body temperature of a mouse could be delayed if theophylline or pentobarbital were given just before the daily rise in temperature.[35] On the other hand the rhythm is set ahead (advanced) if theophylline is given just after the daily high in temperature; but pentobarbital given at this time does not advance the rhythm. Thus drugs can be thought of as chronobiotics and may be used to alter the biological time structures by rephasing the circadian rhythm - this too may be exploited in chemotherapy and radiotherapy. How meal timing and/or drugs may be used in man for optimizing the effect of radiation therapy or chemotherapy timed according to his rhythms awaits further study.

After reviewing a number of investigations designed to explore the mechanisms of rhythmic responses to drugs and toxic materials, we conclude that they have raised many cogent questions; but a discussion of this problem is beyond the scope of this paper. The subject has been treated more in detail elsewhere.[3] In general, these studies caution against generalizing about the causative mechanism of chronopharmacological variation. It seems clear that one cannot ascribe circadian variation in response to drugs as a simple reflection of an underlying circadian variation in hepatic drug-metabolizing enzymes or microsomal biotransformation enzymes; the enzyme or enzyme system of the target organ also may be rhythmic and must be considered.[3]

Many attempts have been made on mammals to elucidate the mechanism of rhythmicity. Among these were studies employing the classic endocrinological approaches of adrenalectomy, hypophysectomy, cerebral ablation, pineal-

ectomy, etc. In spite of these studies and others, no single regulator has been found to account for the control of all rhythmic variables. *Halberg* has shown that certain aspects of 24-hour periodicity are controlled by the adrenal gland, and he suggested that the adrenal cortical cycle deserves serious consideration as the mechanism possibly underlying adaptation to the daily routine.[36,37] The mechanism of circadian control remains an important and fundamental question that will continue to be the subject of intense research.[38]

It is hoped that even without full knowledge of the basic mechanism of molecular control of rhythms or the reasons for variation in response to drugs, data can be produced by rather simple protocols that will have important practical applications in clinical medicine.

Acknowledgement: Our thanks to Dr. Frank Sturtevant, G. D. Searle & Co., Chicago, for critical comments. Supported in part by NIH Grant #CA 14388.

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Section VI - Topics in Chemistry

Editor: R. E. Counsell, University of Michigan, Ann Arbor, Michigan

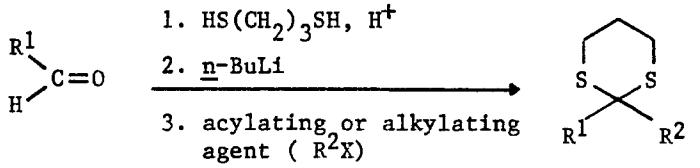
Chapter 27. Reactions of Interest in Medicinal Chemistry

D. L. Venton and Matthias C. Lu, Department of Medicinal Chemistry, College of Pharmacy, University of Illinois, Chicago, Illinois 60680

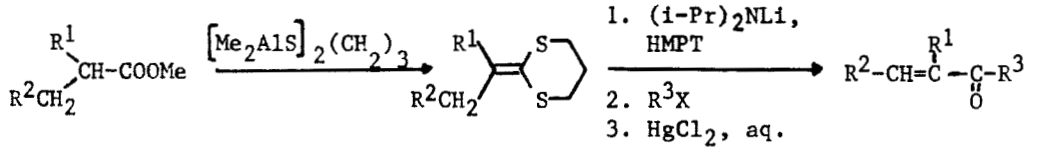
Publication of several useful reference books of general interest in 1975 should be noted: Organic Syntheses Via Boranes by H. C. Brown; Organic Compounds, Reactions and Methods, volume 23, edited by B. A. Kazanskii, I. L. Knunyants, M. M. Shemyakin, and N. N. Mel'nikov, translated from Russian by B. J. Hazzard; volume 13 of Advances in Organometallic Chemistry, edited by I. G. A. Stone and R. West; volume 18 of Advances in Heterocyclic Chemistry, edited by A. R. Katritzky and A. J. Boulton; volume 22 of Organic Reactions, edited by W. G. Dauben; volume 54 of Organic Synthesis, edited by R. E. Ireland; and volume 29 of Synthetic Methods, edited by W. Theilheimer S. and A. G. Karger.

Reviews - Methods for the synthesis of α-methylene lactones,[1,2] and the utility of sodium cyanoborohydride as a selective reducing agent[3] were reviewed.

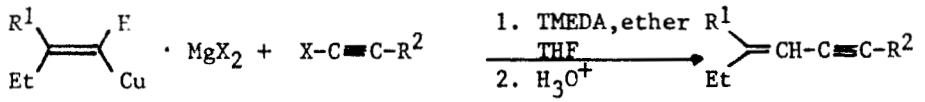
C-C Bond Formations - Metalated dithianes find extended use as synthetic intermediates. Readily prepared from aldehydes, the metalated intermediates react with a great variety of non-enolizable acylating reagents such as carbon dioxide, ethyl chlorocarbonate, acetylchloride, dimethylformamide, and ethyl cyclohexane-carboxylate. They are also rapidly alkylated with primary alkyl iodides or with alkyl or benzylic halides at low temperature to give the thioacetals of higher aldehydes.[4]



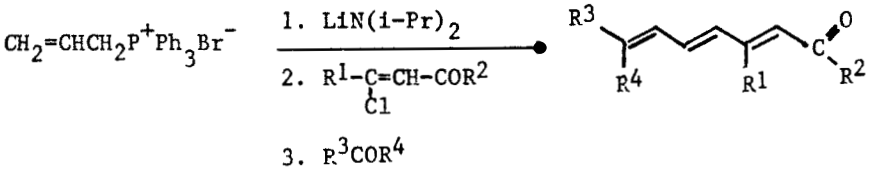
Easily prepared bis/dimethylaluminum/-1,3-propanedithiolate reacts with a variety of methyl esters to produce the ketene thioacetals. Metallation in situ gives the corresponding alkyl anions which react smoothly with alkyl halides to give the 2-alkylated-1,3-dithianes which are converted to the α,β-unsaturated ketones with mercuric chloride.[5]



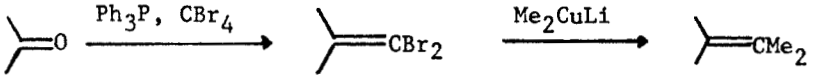
Vinyl copper compounds prepared in the presence of magnesium halides react smoothly with a variety of 1-halo-1-alkynes under very mild conditions to give the conjugated enynes in uniformly high yields.[6]



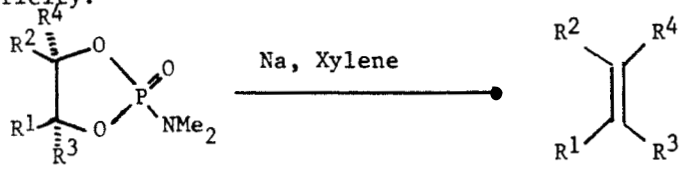
Allylidinetriphenylphosphorane [easily obtained from allyltriphenylphosphonium bromide by treatment with strong base] reacts with chloroacrylate esters and related Michael acceptors to give polyene compounds which, upon quenching with excess aldehydes or ketones give compounds having at least three double bonds conjugated with a carbonyl function.[7]



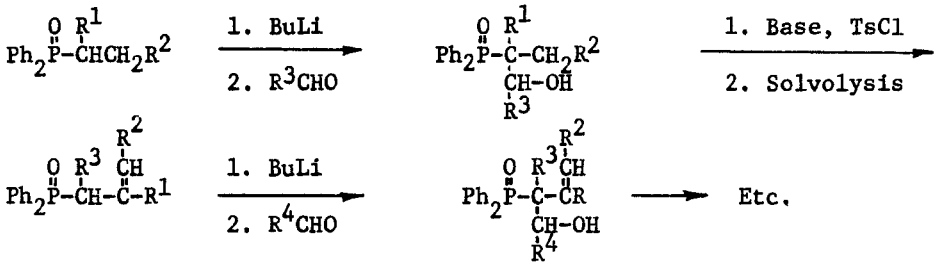
An efficient method for isopropylidination of ketones allows entry into tetraalkylalkenes, usually difficult to prepare by other methods.[8]



Cyclic phosphoric amide derivatives of vicinal glycols are converted to olefins in good yields via reductive syn elimination with moderate stereospecificity.[9]



Although somewhat limited in demonstrated scope, the use of the diphenylphosphinoyl group, $Ph_2P(O)$, to activate a series of carbon-carbon bond forming reactions suggests some interesting synthetic possibilities. In this new approach to successive C-C bond formations the $Ph_2P(O)$ group is moved along the developing carbon framework by solvolysis of the intermediate tosylate derivatives.[10]



Organometallic Reagents - To avoid side reactions resulting from the presence of Cu(II) compounds and other metal salt impurities found in the Cu (I) salts used to form lithium organocuprate reagents, use of the easily prepared, crystalline complex, Me_2S-CuBr, is recommended.[11] This complex is readily soluble in mixtures of Me_2S and ethereal solvents, and the sulfide ligand, Me_2S (bp 37°), is easily separated from reaction products.

Monolithium acetylide and vinyllithium react rapidly with trialkylboranes to produce the lithium ethynyl- and ethenyltrialkylboranes, respectively. Protonation of these species with concentrated hydrochloric acid at low temperature provides directly the borane species with the opposite regiochemistry from that realized in hydroboration of terminal acetylenes or olefins. This gives for the first time a convenient synthesis of the Markovnikov boranes.[12]

Trans-1-tri-n-butylstannyl-1-propene-3-tetrahydropyranyl ether, available in one step from propargyl tetrahydropyranyl ether, allows generation of vinylic nucleophiles; these reagents can be used for the extension of chains, the addition of a vinylic appendage by conjugate addition to an enone, or for ring formation.[13]

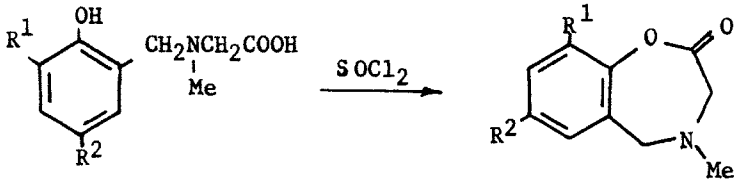
Alcohols and Halides - Several new reagents for the selective bromination of C-H α to a carbonyl function have been reported.[14,15] One of these, 2-carboxyethyltriphenylphosphonium perbromide, is a stable crystalline salt which is easily prepared and is indifferent to double bonds.[15]

An efficient procedure for the α-halogenation of acyl halides has been demonstrated using N-bromosuccinimide (NBS), N-chlorosuccinimide, and molecular iodine. Thionyl chloride was found to be the most effective solvent for all these halogenation reactions. NBS is not only easy to handle but also α-brominates more rapidly and efficiently than the previous molecular bromine procedures.[16]

Treatment of organic halides by the hydridotetracarbonylferrate anion $HFe(CO)_4^-$ affords a simple, mild and stereospecific method for dehalogenating organic halides. Use of $DFe(CO_4)^-$ results in stereospecific monodeuteration.[17]

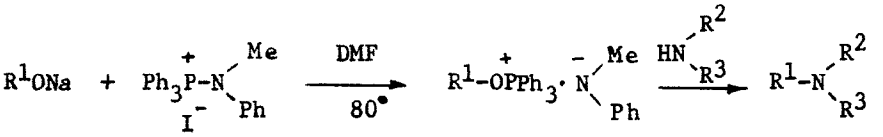
Dialkylaminosulfur trifluorides are easy to handle fluorinating agents which are particularly useful for replacing hydroxyl groups in alcohols and carbonyl oxygen atoms in aldehydes or ketones. They are especially useful for reaction with alcohols or aldehydes which are sensitive to acidic conditions.[18]

Amines and Nitriles - Amino acids have been found to participate as the amine component in the Mannich reaction with both ketones and phenols. The phenolic Mannich bases may be cyclized with sulfuric acid to 1,2-dihydro-4(3H)-isoquinolones. In the presence of thionyl chloride the same Mannich bases undergo lactonization to benz[f]-1,4-oxazepin-4(3H)-ones.[19]



Basic treatment of O-2,4-dinitrophenyloximes, easily obtained by condensation of aldehydes with O-2,4-dinitrophenylhydroxylamine, leads to high yields of nitriles under mild conditions.[20]

Sodium alkoxides, prepared by treatment of the respective alcohols with sodium hydride in dimethylformamide (DMF), react with N-methyl-N-phenylaminotriphenylphosphonium iodide to give phosphonium salts which are converted, without isolation, to the secondary and tertiary amines by stirring with various amines in DMF solution at 80° for two hours. The advantages of this new method are: mild reaction conditions with high versatility and efficiency as well as selectivity.[21]



The Lindlar catalyst, previously employed for the selective reduction of acetylenes to cis-olefins, can also be used to catalyze the selective hydrogenation of azides to amines in the presence of double bonds and carbonyl groups.[22]

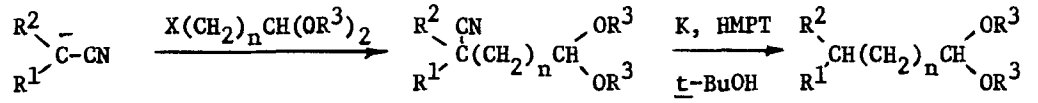
Carboxylic Acids and Derivatives - A general, high yield method for the preparation of α-hydroxycarboxylic acids involves the reaction of ketene bis(trimethylsilyl) acetals with m-chloroperbenzoic acid.[23] Other methods recently reported for the preparation of α-hydroxycarboxylic acids include the aeration of lithiated carboxylic acids,[24] synthesis by use of the Pummerer reaction,[25] and an improved procedure using activated zinc in the Reformatsky reaction.[26]

Hydrolysis of amides to acids by aqueous sodium peroxide (in less than 2 hr. at 50-80°) in high yield and with little decarboxylation of the acid has been reported.[27]

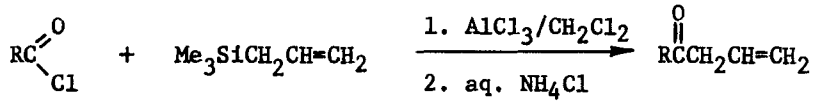
Silver carbonate on Celite oxidizes primary 1,4-, 1,5-, and 1,6-diols to lactones in high yield. This method is superior to previously described syntheses involving dehydrogenations at elevated temperatures and other oxidations which afford many side products.[28]

Aldehydes and Ketones - A facile synthesis of aldehydes involves treatment of the corresponding carboxylic acids with chloroformates in the presence of triethylamine. Reaction of the resulting anhydrides with disodium tetracarbonylferrate [$Na_2Fe(CO)_4$] in dry THF at room temperature and subsequent quenching of the reaction mixture with acetic acid gives the desired product.[29]

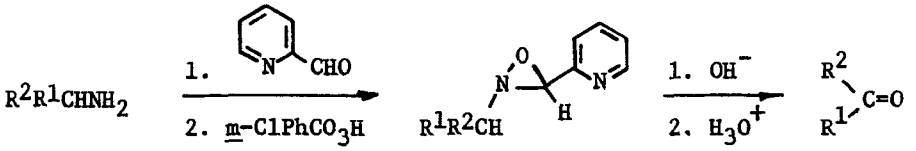
An elegant method for the preparation of β and γ alkyl aldehydes or acids involves the cyano function as an activating group. This method is of particular value, as removal of a cyano function can be effected by reduction in HMPT in excellent yields.[30]



Alkyl and aryl allyl ketones can easily be prepared by the reaction of allyltrimethylsilane with acyl chlorides in the presence of $AlCl_3$.[31]

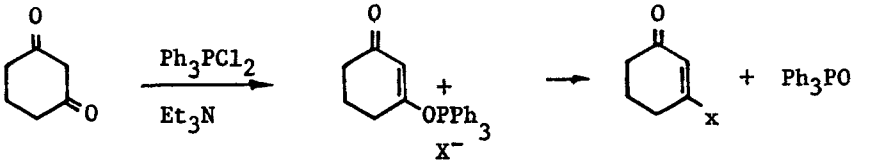


Ketones can also be obtained by oxidative elimination of amines via oxaziridines;[32]



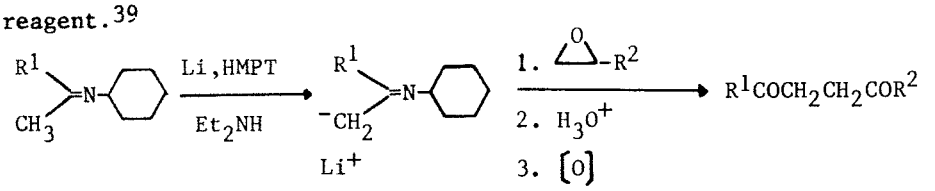
by oxidative decyanation of secondary nitriles;[33] or by oxidative decarboxylation of α,α'-disubstituted carboxylic acids.[34,35]

A new, efficient synthesis of cyclic β-halo-α,β-unsaturated ketones from β-diketones has been reported. Treatment of 1,3-cyclohexanedione with freshly prepared triphenylphosphine dihalide and triethylamine at room temperature affords previously unattainable 3-bromo- and 3-iodo-2-cyclohexen-1-one in 97% and 72% yield, respectively.[36]



Numerous reports appeared in 1975 describing the synthesis of 1,4-diketones, which are synthetically useful but not generally available compounds.[37-42] For example, addition of dimetalacetylenides to the aldehyde, catalytic reduction of the triple bond, and subsequent oxidation of the diol gives the 1,4-diketones in 20-60% overall yield.[37] Unsymmetrical 1,4-diketones are readily prepared from metalated ketimines, by condensation with an oxirane, and subsequent oxidation of the resulting γ-ketoalcohols by Jones

reagent.[39]



Several new or improved procedures for the synthesis of β-hydroxyaldehydes,[43] β-ketoaldehydes,[44] α,β-unsaturated ketones,[45,46] α-ketoesters and unsymmetrical 1,2-diketones,[47] β,γ-unsaturated aldehydes,[48] and γ,δ-unsaturated ketones[49] have been reported.

Oxidations - Organoboranes are oxidized efficiently by trimethylamine N-oxide dihydrate to the corresponding alcohols in excellent yields (94-100%). The reagent is exceptionally mild, permitting the oxidation of a wide variety of functionally substituted organoboranes.[50] A convenient preparative method for stereospecific hydroxylation of tertiary carbon atoms by ozonation of saturated compounds adsorbed on silica gel has been described.[51]

Pyridinium chlorochromate, $C_5H_5NHCrO_3Cl$, a readily-available, stable reagent offers certain advantages over the more classical Collins reagent.[52] Mild oxidation of alkyl halides directly to acylhydrazone protected aldehydes is of synthetic utility in the case of labile aldehyde preparation.[53]

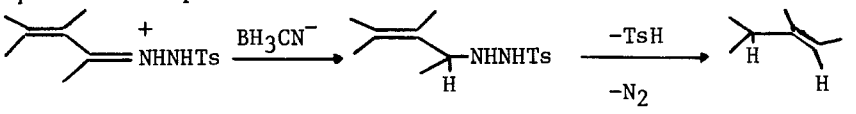
The oxidation of terminal olefins by Jones reagent in the presence of a catalytic amount of Hg(II) gives good yields (77%) of the corresponding methyl ketones.[54]

A new route to epoxides which involves reaction of phenyl selenoacetals and carbonyl compounds has been reported,[55] and a general method has been developed for the synthesis of non-K-region arene oxides of polycyclic aromatic hydrocarbons.[56]

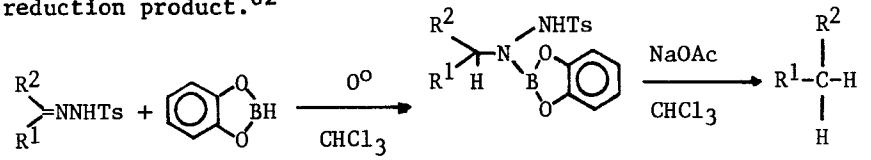
Reductions - Aldehydes are smoothly reduced to alcohols in the presence of ketones with stoichiometric amounts of sodium triacetoxyborohydride,[57] lithium tri-t-butoxyhydroaluminate, sodium borohydride or lithium borohydride.[58] Similarly, dehydrated Woelm chromatographic alumina treated with 2-propanol reduces structurally diverse aldehydes cleanly and rapidly (< 2hrs) to the corresponding alcohols at room temperature in 65-88% yields. With $(CH_3)_4CDOH$ on alumina, aldehydes may be converted to the corresponding 1-deutero-alcohols.[59]

Reduction of α,β-unsaturated aldehydes and ketones with 9-borabicyclo-[3.3.1]nonane(9-BBN) proceeds selectively and cleanly to the corresponding allylic alcohols in excellent yield in the presence of many other functional groups.[60] The combination of $NaBH_3CN$ in acidic DMF-sulfolane provides an effective and convenient system for the reduction of α,β-unsaturated carbonyl tosylhydrazones specifically to the corresponding alkenes with migration of the double bond most likely via a 1,5-sigmatropic shift. The reduction proceeds stereoselectively to furnish the E geometric isomer as

the predominant product.[61]



Ketones may be converted to the corresponding methylene derivatives via their tosylhydrazones under mild conditions which involve the reduction of tosylhydrazones with catecholborane followed by decomposition of the reduction product.[62]



On reaction with tributylstannane, O-cycloalkylthiobenzoates and O-cycloalkyl-S-methyldithiocarbonates, derived from secondary alcohols, give good yields of the corresponding hydrocarbons. This procedure is of particular interest in the synthesis of deoxysugars.[63]

Hydrogenation of quinoline, isoquinoline, acridine, 2-or 4-phenylpyridine over platinum oxide in strong acid (12N-HCl, 12N-H_2SO_4 or CF_3COOH) leads to selective hydrogenation of the benzene ring.[64]

The reduction of α,β-unsaturated nitriles to saturated nitriles is achieved with Mg in MeOH. This method is superior to catalytic hydrogenation in its selective reduction of conjugated double bonds in the presence of non-conjugated ones.[65]

9-BBN and n-butyllithium complexes in hexane, provide a new type of reducing agent for the selective reduction of tertiary alkyl, benzyl and allyl halides to afford the corresponding hydrocarbons in excellent yields without concomitant attack on the secondary, primary and aryl derivatives.[66]

Protective Groups and Their Removal - Levulinic ester represents an attractive protecting group for alcohols, especially for carbohydrates, nucleosides and steroids, when selective protection and deprotection of alcohols in the presence of other protecting groups such as acetals, trityl ethers and benzoyl esters are desired.[67,68] The removal of levulinates can be accomplished with $NaBH_4$,[67] or by hydrazine,[68] and the reisolation of alcohols is facile since the by-product is water soluble.

Methylthiomethyl (MTM) protecting group offers an excellent alternative for the selective protection of primary alcohols. The MTM ether function is stable to basic and nucleophilic reagents e.g., NaH, RLi or NaOR. In addition, it is fairly resistant to acid-catalyzed cleavage. Thus selective removal of other protecting groups such as acetonides, and tetrahydropyranyl groups can be accomplished. The conversion of the MTM ethers to the corresponding alcohols can be effected cleanly by reaction with $HgCl_2$ in CH_3CN or with $AgNO_3$ in THF-H_2O at 25°.[69]

N,N'-Carbonyldiimidazole is an excellent reagent for the protection

of vicinal diols via formation of the cyclic carbonates in very high yields. The advantage of this method over conventional methods (i.e., phosgene or alkyl chlorocarbonates) is that the only by-product, imidazole, can be easily removed because of its water solubility.[70]

Methylthiotrimethylsilane reacts spontaneously at 0° with aldehydes and ketones to give dimethylthioketals in excellent yields without the apparent requirement of acid catalysis.[71]

New Syntheses - Preparation of 5,8,11-dodecatriynoic acid via Grignard coupling of 1-bromo-2,5-hexadiyne and 5-hexynoic acid has been described. The triynoic acid has been used as an intermediate in a new synthesis of arachidonic acid and of novel methyl-branched arachidonic acids.[72]

A new synthesis of coumarins, by direct cyclization of α-cyano-O-methoxycinnamates in sulfuric acid appears quite general.[73]

3,5-Dibromo-4-oxo-2,2,6,6-tetramethyl-1-piperidinyloxy is a highly reactive acylating agent for amines, converting primary and secondary amines, amino-acid esters and amino-alcohols to crystalline amides. This method offers considerable advantage over other tedious literature methods and represents a short-cut method to spin-labelled amides.[74]

X= $-NR_2$, -OH, -Cl, -OEt

Fatty acid esters have been synthesized in good yield by reaction between copper (I) and primary or secondary Grignard reagents and esters of primary iodoalkylcarboxylic acids. This method provides the most direct route presently available to a variety of representative classes of simple fatty acids.[75]

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Chapter 28. Total Synthesis of β-Lactam Antibiotics

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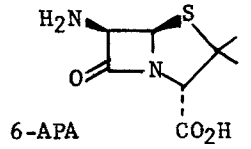
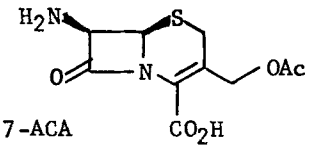
Introduction - Penicillins and cephalosporins constitute one of the most widely prescribed classes of antibacterial agents in clinical practice, largely because of their unique safety and the availability of a large family of semi-synthetic analogs with altered pharmacodynamic properties and antibacterial spectrum. Indeed, the prowess of the medicinal chemist in modifying the parent antibiotics has provided many of the most cited examples of successful drug modification. Historically, most of these derivatives were made semi-synthetically, i.e. by acylation of 6-aminopenicillanic acid (6-APA) or 7-aminocephalosporanic acid (7-ACA) or by displacement of the 3'-acetoxy group of the cephalosporins. More recently, the quest for novel structural types has required more profound transformation of the basic nuclei themselves. Direct substitution of the nucleus, especially onto the 7α position of the cephalosporins, or disruption of the thiazolidine or dihydrothiazine rings followed by closure to a new ring system have provided examples of structures not available by fermentation and semi-synthetic modification. However, it is readily apparent that many attractive structures can be made most readily by total synthesis and during the past decade considerable activity has been seen in this area of β-lactam research.



6-APA

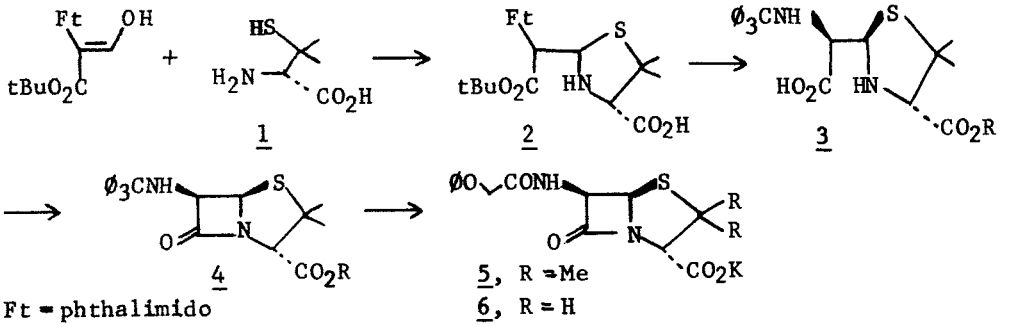


7-ACA

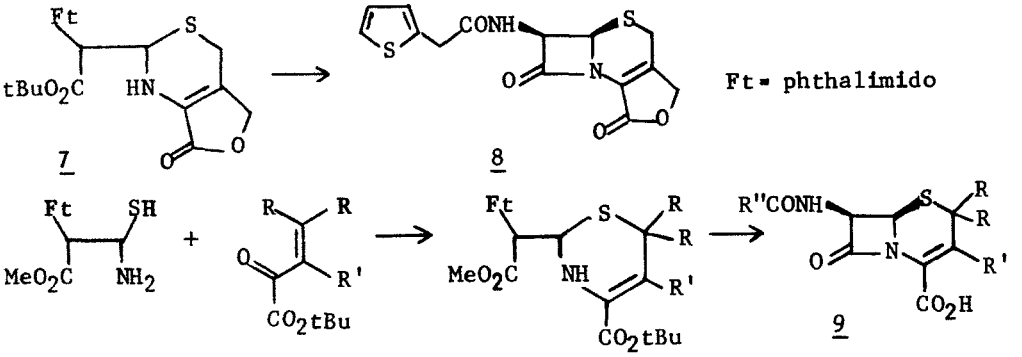
Only cell-wall biosynthesis inhibitors are likely to possess the remarkable safety of this class of antibiotics. With few exceptions, previous work has indicated at least four requirements for antibacterial activity as transpeptidase inhibitors: 1) a reactive bicyclic β-lactam, activated either by strain or electronic factors, 2) an amido function α to the β-lactam carbonyl, 3) proper stereochemistry, the sulfur atom must be cis to the amide nitrogen, and 4) a free carboxyl group on the carbon atom adjacent to the azetidinone nitrogen. Analogs prepared by total synthesis afford an unusual opportunity to further modify and refine these requirements. This review will only consider totally synthetic analogs that meet these requirements, and not model compounds or simpler analogs which have been adequately reviewed[1] elsewhere. Likewise, the classic penicillin synthesis by Sheehan, the Woodward cephalosporin work, and Roussel-Squibb synthesis of desacetylcephalothin lactone have been treated several times.[2-4] Instead, emphasis will be placed on more recent developments.

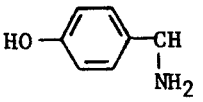
Analogs Based on the Sheehan Penicillin Synthesis - Although the literature contains two earlier reports of penicillin total synthesis with final step yields of 0.1% or less, the first classic synthesis is that of Sheehan.[5] This synthesis has been reviewed several times, yet several

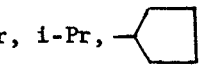
key transformations showing the essence of that pioneering work deserve comment. Although the γ-isomer of 2 is the major condensation product, it can be equilibrated to the desired α-isomer with pyridine. Carbodiimide mediated closure of penicilloic acid 3 gave penam 4 which was converted to penicillin V (5). By using D-cysteine in place of 1, Hoogmarten, Claes, and Vanderhaeghe[6] prepared bisnorpenicillin V (6). 6 is generally less potent than the parent penicillin and is just as sensitive to β-lactamase.



The Sheehan strategy has been extended to the total synthesis of desacetylcephalothin lactone (8)by researchers at Roussel[7] and Squibb.[8] Merck chemists[9] have developed a more efficient synthesis of intermediate 7. Recently,[10] a further modification of their basic scheme allowed the Roussel workers to prepare a series of analogs of type 9. No biological activities are available from the abstract.



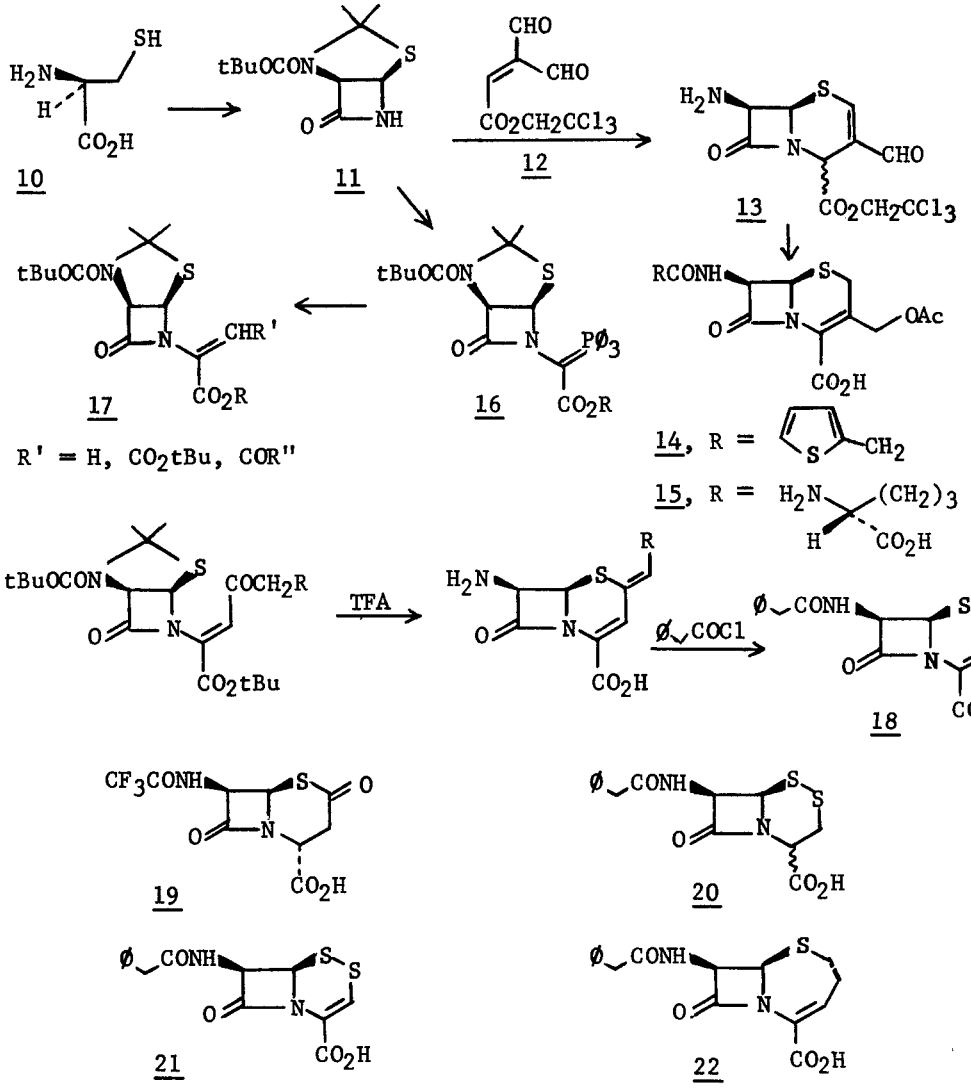
R" =  , ØCH(NH2) ,  R = Me, R' = H

R = H, R' = Me, Et, n-Pr, i-Pr,  , Ø, , CH_2CH_2OAc

The Woodward - Ciba-Geigy Approach - The starting material in the Woodward approach[11] was L-cysteine (10), thus fixing the chirality of the final products as in the natural cephalosporins. By a series of ingenious and stereospecific transformations, 10 was converted to a key intermediate, fused thiazolidine 11. Compound 11 reacted with the highly electrophilic dialdehyde 12 and provided bicyclic intermediate 13 upon acid treatment.

Subsequent manipulations afforded cephalothin (14) and cephalosporin C (15).

The Woodward - Ciba-Geigy group early recognized the potential of intermediate 11 for the synthesis of novel bicyclic β-lactams. The Wittig reagent 16, prepared by sequential reaction of 11[12] with a glyoxylic ester, thionyl chloride, and triphenyl phosphine, reacted with aldehydes to yield olefins 17. These materials were converted to the unnatural cephalosporin analogs 18-21.[13] The process is represented in its simplest form for the preparation of 18.

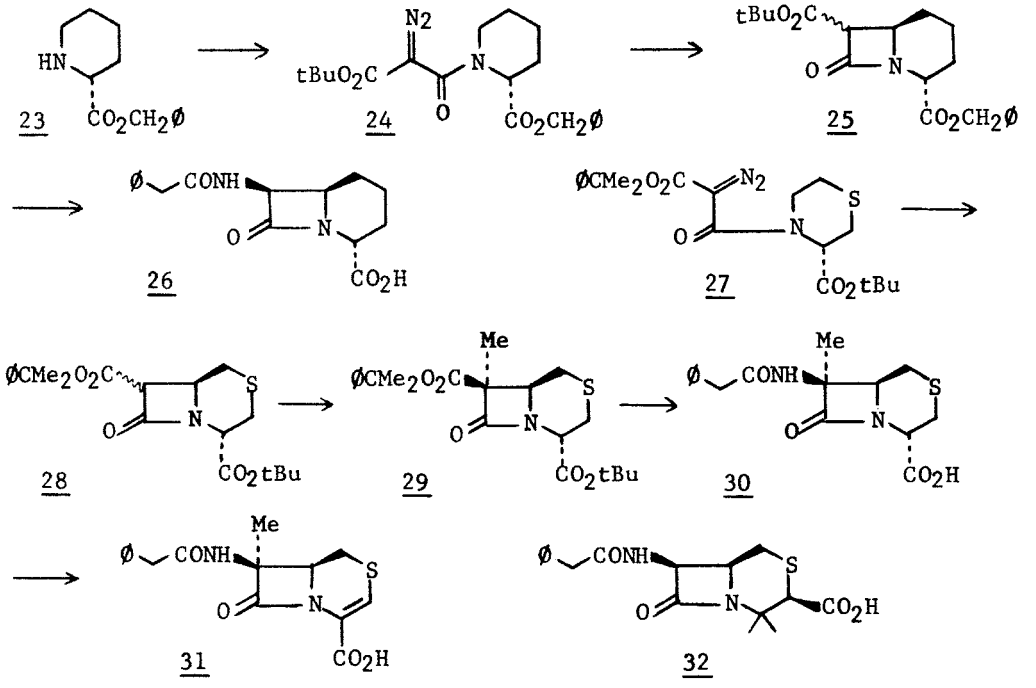


Some of the compounds of type 18, where R represents alkyl, haloalkyl, and a number of substituted phenyl derivatives, showed activities against staphylococci in vitro comparable to or better than penicillin,

but their in vivo activity was disappointing. Compound 19 showed a low order of antibacterial activity and both isomers of 20 showed some antibacterial activity in vitro. This activity[13] was much increased in the unsaturated analog 21. Compound 22, which was prepared[14] by yet another variant of the phosphorane route, was inactive in vitro versus both gram positive and gram negative bacteria at 100 μg/ml.

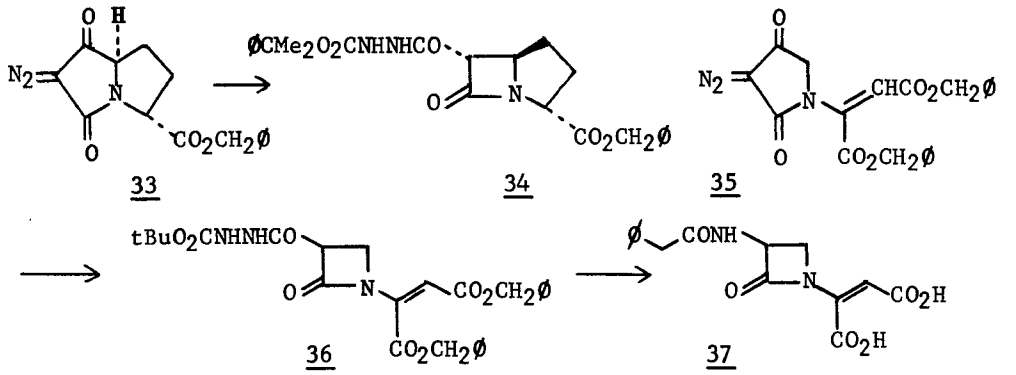
The Lowe Approach - Lowe and coworkers[15] have developed two general methods which allow entry into a variety of nuclear analogs of the penicillins and cephalosporins. The first approach, which is based on an earlier observation of Corey and Felix,[16] utilizes the photolysis of a diazoamide from a cyclic imino-acid ester to give a fused β-lactam-heterocycle system.

Coupling of benzyl D-pipecolate (23) with t-butyl hydrogen malonate followed by diazo exchange with tosyl azide gave the diazo intermediate 24. Photolysis of this compound produced a 1:2 mixture of cis- and trans-diesters 25. A multi-step, indirect Curtius rearrangement procedure was developed for conversion of the cis-t-butyl ester group into a phenylacetamide side-chain with retention of configuration. Hydrogenolysis of the benzyl group provided the nuclear analog 26.[17] Similarly, photolysis of diazoamide 27 produced bicyclic β-lactam 28 as a 7:3 cis-trans mixture. Since the desired cis-isomer of 28 exhibited pronounced stereochemical lability, an α-methyl substituent was introduced to form the stereochemically stable system 29. Subsequent transformation afforded the saturated and unsaturated analogs 30 and 31.[18] Analog 32 was also prepared by photolysis of the corresponding precursor diazoamide.[19]

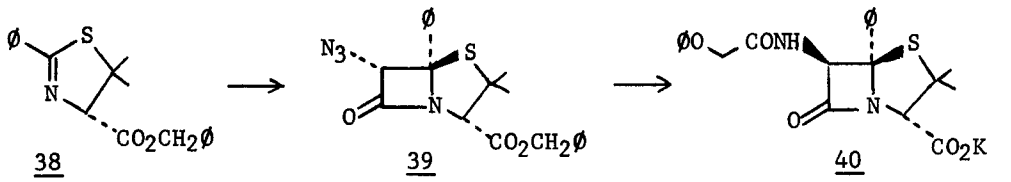


No antibacterial activity was observed when compounds 26, 30 and 31 were tested at 1 mg/ml against Staphylococcus aureus, Salmonella typhi and Alcaligines faecalis. Analog 32 showed no activity against S. aureus and A. faecalis at 1 mg/ml.

In their second approach, the Oxford group made use of the photolytic Wolff rearrangement of diazopyrrolidinediones to generate β-lactams. For example, photolysis of 33 in the presence of α,α-dimethyl benzylcarbazate afforded 34. This product was judged too unstable for the subsequent synthetic manipulations required to convert it into a nuclear analog of penicillin.[20] Monocyclic diazopyrrolidinedione 35 was likewise photolyzed to yield azetidinone 36. Transformation of the hydrazide into the phenylacetamide side-chain followed by hydrogenolysis of the benzyl ester gave diacid 37. The acid[21] showed no inhibition of the growth of S. aureus, A. faecalis, or S. typhi at 1 mg/ml.



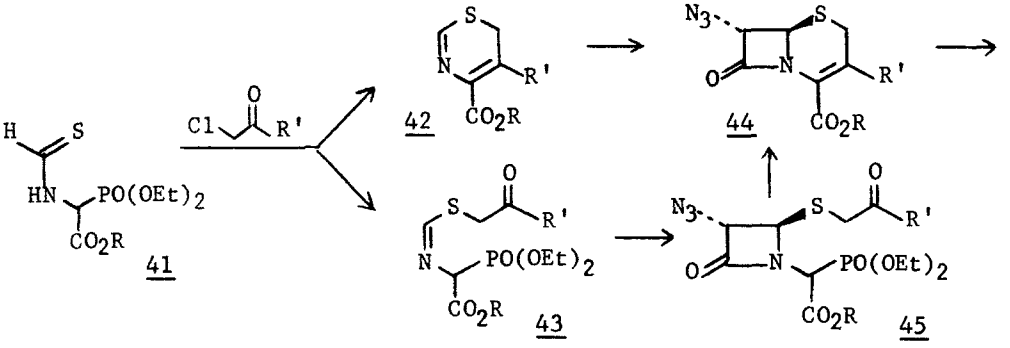
The Acid Chloride-Imine Cycloaddition Approach - One of the most direct routes to bicyclic β-lactams is based on the well-known cycloaddition of ketene precursors with amines.[22] This method has been developed by Bose and co-workers[23] in a total synthesis of (±)-methyl 6-epipenicillin V. In an extension of earlier work, Vanderhaeghe and Thomis[24] utilized this approach to prepare 5-phenyl penicillin V (40). Although cycloaddition of D-2-phenylthiazoline 38 with azidoacetyl chloride gave the 6-epi penam 39, the stereochemistry could be inverted in a later stage of the synthesis. The resulting potassium salt of 40 had less than 1/1000 of the activity of penicillin V when tested against Staphylococcus aureus.



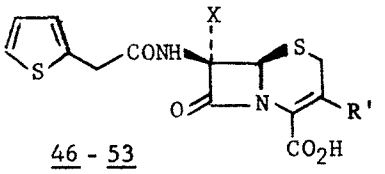
The Merck group has utilized the cycloaddition approach in a simple and unique synthesis capable of providing both pharmacologically important cephalosporin antibiotics and nuclear analogs of the basic cephem system. The starting material, α-thioformamido-phosphonoacetate 41,[25] condensed

with α-chloroketones to yield either 1,3-thiazines 42 or thioimidates 43. Cycloaddition with azidoacetyl chloride afforded either cephems 44 or azetidinones 45, the latter being cyclized to 44 with base. Subsequent transformations provided a series of racemic cephalosporin derivatives.[26-29] Crucial to the success of this route was the development of a novel kinetic epimerization method[30] to correct the stereochemistry at position 7 in the cephem system. The epimerization method was also applied in a total synthesis of penicillin G.

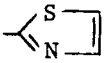
Unexpectedly, the facile lactonization of 3'-methyl analog 50 results in a half life ($t_{1/2}$) of ca. 20 mins. in aqueous solution at 37 making this compound too unstable for consideration as an antibacterial agent. Analogs 51-53 showed reduced gram positive and poor gram negative activity when compared with cephalothin (47).



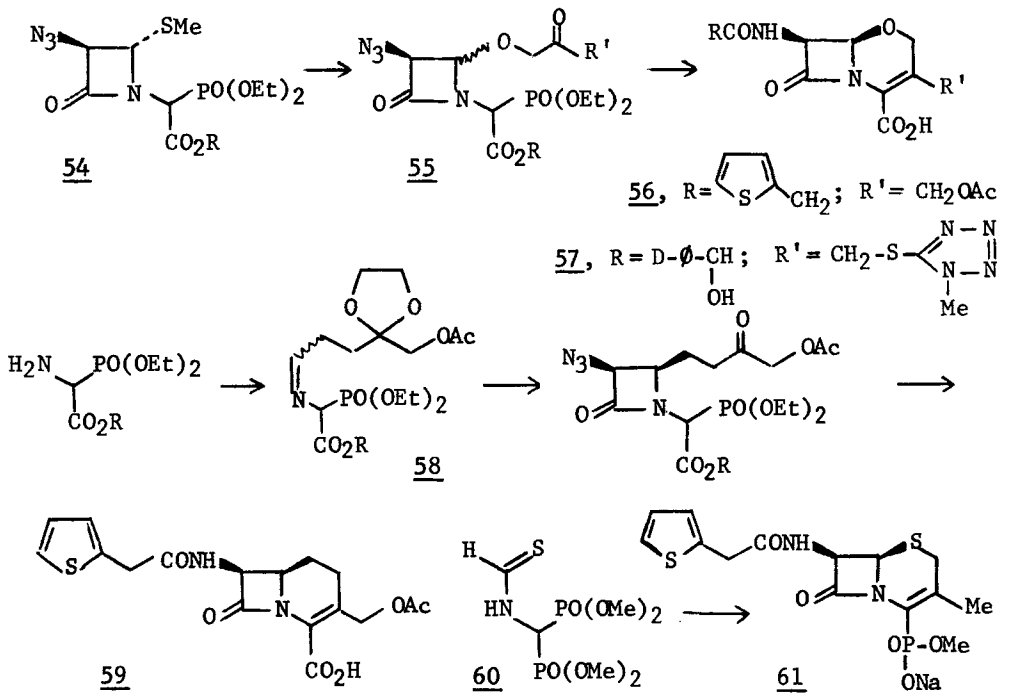
41 42 43 44 45



46 - 53

Compd.	X	R^1
46	H	CH_3
47	H	CH_2OAc
48	OCH_3	CH_2OAc
49	OCH_3	CH_2OCONH_2
50	H	$CHCH_3OAc$
51	H	C_6H_5
52	H	p-CO_2Me-C_6H_4
53	H	

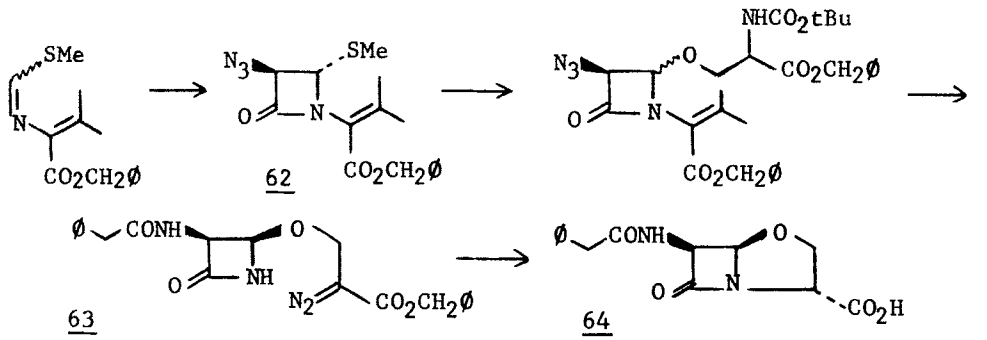
The generality of the Merck approach is illustrated by the preparation of nuclear analogs. Methylation of 41 followed by cycloaddition provided azetidinone 54. Replacement of the methylthio substituent by halogen gave a reactive intermediate which solvolyzed in the presence of ketoalcohols affording oxo derivatives 55. Transformations as already outlined gave a series of racemic 1-oxa-dethiacephalosporins, examples of which are 56 and 57.[31,32] In a similar manner, Schiff base 58 provided racemic 1-carbadethiacephalothin (59).[33] Replacement of the carboxyl function in 41 by phosphono allowed entry into the 4-phosphono cephem series, as represented by the conversion of 60 to 61.[34]



The effects of isosteric changes of the sulfur atom of cephalosporin upon in vitro activity is readily apparent from the observation that 47, 56, and 59 are essentially equipotent. These data provide the first indication that the sulfur atom is not essential for activity. Indeed, (±)-1-oxa-dethiacephamandole (57) is at least twice as active as its sulfur counterpart against 8 pathogens (including both gram positive and gram negative bacteria). Replacement of the 4-carboxyl function by phosphono as in 61 resulted in a compound less active in vitro than the corresponding cephalosporanic acid derivative.

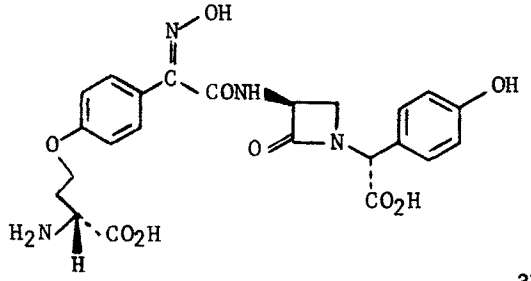
A new approach to the construction of a bicyclic β-lactam system was described in the total synthesis of penam analog 64.[32] This synthesis also employs the azidoacetyl chloride-imine annelation procedure to produce azetidinone 62. The introduction of the oxygen atom was accomplished in a manner analogous to the previously described 1-oxa-dethiacephem synthesis using a suitably blocked serine derivative. After subsequent reduction, phenylacetylation, deblocking of the nitrogen atoms and diazotization, the crucial intermediate 63 was obtained. Rhodium acetate generation of the carbene results in the oxa-dethiapenam ring system. Hydrogenolysis afforded racemic 64. Although bioactive in vitro, analog 64 proved too unstable ($t_{\frac{1}{2}} < 20$ mins at 37°) for accurate testing.

Syntex chemists[35] have also utilized the acid chloride-imine approach in their total synthesis of (±)-desacetylcephalothin lactone. Although this method of β-lactam construction was not employed, it ought to be mentioned for the sake of completeness that Kishi's group[36] has synthesized cephems by an entirely different procedure. Neither of these routes have

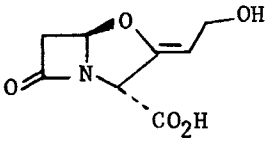


been applied to analog synthesis.

Conclusion - The preceding examples demonstrate that total synthesis is an effective way to make analogs of quite respectable and even enhanced activity over the naturally occurring nuclei. Certainly the bioactivities of some of the totally synthetic compounds have altered our current thinking on the effect of structure upon activity in this field. Indeed it might be mentioned that another source of truly different structures is natural product isolation and recently two novel β-lactam antibiotics (see below) have been discovered.



Nocardicin A (FR1923)[37]



Clavulanic Acid[38]

As the contribution of the various structural features to activity is further clarified, even more novel structures with unique activities will be synthesized. As a result, the use of total synthesis as a means for making β-lactam analogs will probably increase. With the advent of more efficient syntheses and structurally simpler active compounds, we can look forward to the commercial exploitation of total synthesis methodology.

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Chapter 29. Polymeric Reagents in Organic Synthesis

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I. Introduction and Scope - It has been over 40 years since ion exchange resins were first used.[1] It has been more than 15 years since Merrifield's brilliant concept of polypeptide synthesis on polymer supports was first divulged and tested.[2] However, given the real and potential benefits from this approach, it is surprising that the use of these support materials in general organic synthesis has been so slow in developing.[3-6] It is our intention in this review to limit comment to those applications where the polymer is the reagent, capable of transmuting a monomeric substrate that may or may not be transiently bound to the reagent during the reaction. This, therefore, rules out multistep transformations on polymers such as the polypeptide, oligonucleoside and polysaccharide syntheses[7] as well as the resin controlled reactions described by Patchornik[8-11] and Rapoport.[12-15] In addition, the field of immobilized enzymes (a special field of polymeric reagents) requires its own review and will not be covered here.

II. General Considerations and Polymer Structure - Obviously ion-exchange clays and resins are historically the first of the polymeric reagents. The reactions performed are simple, straight-forward and quantitative. By conducting reactions in two phases (the solution of the substrate and the solvated polymer gel phase) many aspects of the physical manipulation become simpler. Reaction workup, product isolation, removal of reagent by-products, absence of volatility of potentially noxious reagents, easy recovery and recycling of reagent are all important advantages. In the eventual production of a new pharmaceutical the simple removal of reagent by-products could aid in avoiding toxic contaminants.

Choice of the proper polymer matrix is an important factor. Polystyrene, crosslinked with varying amounts of divinylbenzene, is undoubtedly the most studied and most utilized matrix for polymeric reagents.[16] However, for special purposes glass beads[17], polystyrene grafted to teflon[18], acrylate esters[19] and crosslinked dextrans[20] (Sepharose)® have been utilized. This review will concern itself only with the polystyrene type.

Once one has settled on a polystyrene matrix, further decisions must be made since various methods are available for production of the beads. A considerable variation in properties is available depending on (a) percent of divinylbenzene, (b) nature of the solvents involved in the emulsion polymerization and (c) contaminants retained after the polymerization.[16]

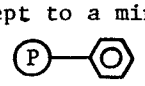
Traditional beads shrink or swell considerably depending on the solvent they are placed in and this seriously affects the availability of reactive sites within the matrix.[21,22] On the other hand, new developments in macroreticular resins have made available matrices with larger pore sizes and minimal shrinking and swelling with changes in solvent

polarity.[23,24]

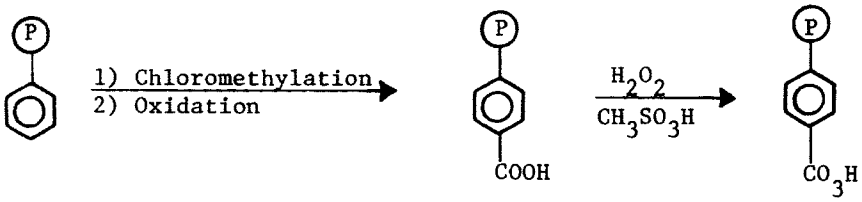
Although it was somewhat naïvely thought that these matrices could serve very efficiently to isolate individual reactions on the matrix, (since there could be little internal movement) this is not the general case, and only under certain conditions can restricted interaction of reactive sites be achieved.[15] It has been shown that matrices with several percent crosslinking have the capability for very significant internal mobility.[25] However, there is clear indication that successful "high *dilution*" type results can be obtained [8,26-29] under the proper conditions.

In general, the matrix can be functionalized using typical reactions that proceed on a mono-alkylated benzene ring as a model. Examples of chloromethylation[30-32], bromination[33], sulfonation[34], metalation[28,35] and nitration[36] all give rise to reactive intermediates which can be further converted to polymeric reagents as illustrated in the next section. Proper choice of solvent can be critical even when using the macroreticular resins.[28] In addition, pretreatment with solvent prior to adding reagents can significantly affect the degree of substitution.

One of the major problems encountered in the preparation and use of polymeric reagents is the difficulty in analyzing the materials.[15] Many of the highly useful techniques employed in modern day chemistry such as proton magnetic resonance, thin layer chromatography, ultraviolet absorption, and gas chromatography analysis are not applicable to the solid phase. One is left only with solid state infrared and elemental analysis as direct methods. Occasionally direct titration of particular groups is possible, but in many cases the progress of a reaction must be monitored by indirect methods such as the disappearance of certain species from the liquid phase.

III. Applications - In this section we will cover a broad range of reaction types that can be carried out with polymeric reagents applicable to medicinal chemistry. Specific examples will be kept to a minimum so that a comprehensive survey is obtained. The symbol  will be used to designate a polystyrene matrix crosslinked via divinylbenzene.

A. Oxidation - Polymeric peracids have been synthesized and utilized to convert olefins to epoxides with the advantage of greater stability of the peracid and simple regeneration of the reagent.[37] Although feasibility of this approach has been demonstrated, further work is necessary to



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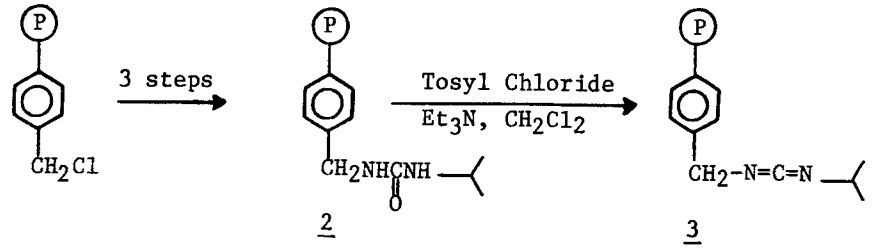
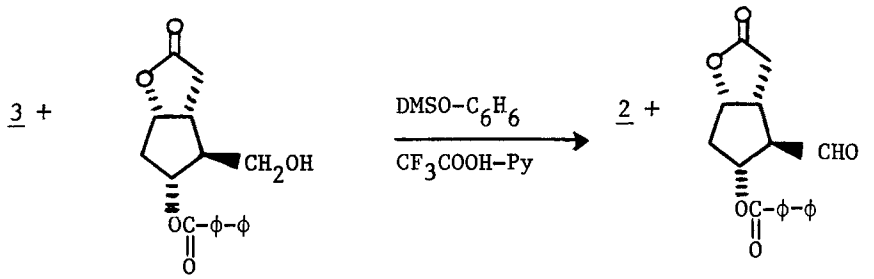
optimize the method. Ideally, one would like to use flow system techniques such that the generated epoxide is carried away from the resulting polymeric acid thus avoiding side reactions.

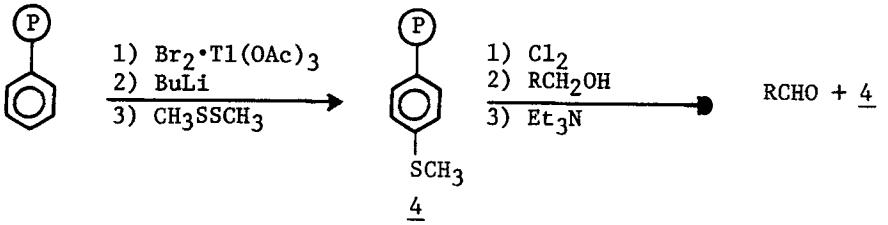
A polymeric carbodiimide 3 has been utilized in a Pfitzer-Moffat oxidation of a highly sensitive prostaglandin intermediate.[38,39] The reaction by-product, the urea 2 can be recycled to the carbodiimide and reused (with some loss of activity). The major advantage here is the





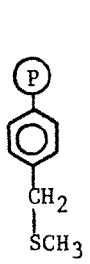
exceedingly simple workup conditions that protect the labile trans *p*-biphenylcarbonyloxy group from elimination.

Polymeric thioanisole 4 has been demonstrated to serve well in the new Corey oxidation method.[28] This is a situation in which the starting



polymeric reagent is regenerated during the reaction and may be reused a number of times. It has also been shown that when 4 is produced at a sufficiently low degree of substitution it can function as a selective reagent in the mono-oxidation of diols. In all of the above applications use of the noxious monomeric thioanisole is avoided with little or no loss in yields. Further reactions utilizing 4 are in progress.

B. Reducing Agents - The polymeric benzyl methyl sulfide 5 reduces hydroperoxides in the same fashion as dimethyl sulfide. The former reagent is useful in the destruction of peroxides and can thus stabilize ethers quite well.[40] Only a single example of a polymeric reducing agent has been reported.[41] The polymeric tin hydride 6 is useful for both reduction of halides and carbonyl compounds, but can be used selectively as shown in the conversion of prostaglandin intermediate 7a to 7b.



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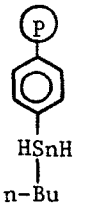

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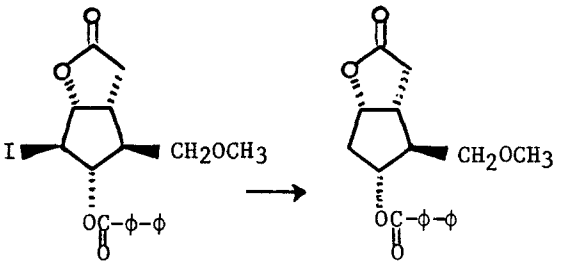


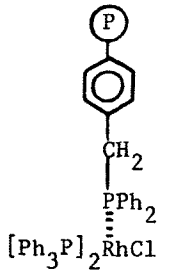
7a 7b

A recent patent describes the use of the thioether reagent 5 to bind BH_3 (up to 2 mmol/gram).[42] The product, stable when protected from air and moisture, effectively serves as a solid source of borane to perform reductions and hydroborations.

C. Catalysts for Photochemical Reactions - Chloromethylated polystyrene has been substituted with rose bengal or fluorescein.[43,44] This product has then been used effectively as a photosensitizer to generate singlet oxygen as a synthetic intermediate. The catalyst can be used repeatedly and isolation of sensitive products (e.g., peroxides) is greatly simplified.

D. Metal Catalysts - The use of various complexed metals in soluble forms has given the chemist a great new array of hydrogenation, hydrocyanation, hydroformylation and dimerization techniques. In many cases, these metals are quite expensive and their complete recovery from a reaction mixture is tedious. By coordination to a polymer-bound ligand, these problems are overcome and in some cases special new properties are encountered.

Grubbs has used the rhodium reagent 8 to hydrogenate substrates at 1 atm of hydrogen.[45,46] There is some indication that access to the catalyst within the bead is controlled by the pore size of the matrix. This in turn



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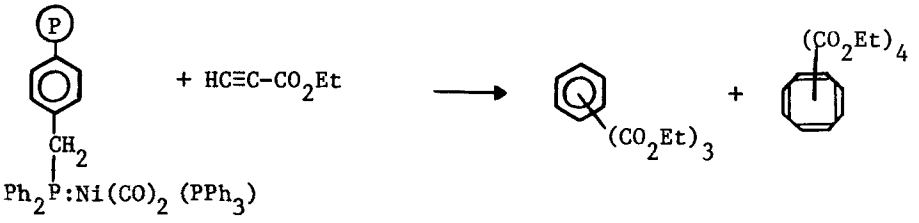
controls the rate of hydrogenation; small mobile olefins are reduced much more rapidly than large rigid molecules. This is in contrast to the

relative rates when using the identical fully soluble catalyst.

A similarly bound nickel carbonyl reagent 9 has been used for catalytic oligomerization by Pittman and Evans.[47]



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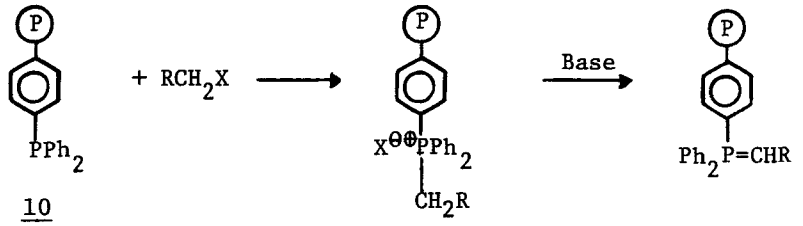
Many other uses of polymerically bound metals are known.[48-53] Perhaps the use of a bound rhodium reagent to catalyze carbonyl reductions by silanes with concomitant asymmetric induction is one of the most elegant.[54] In some cases, greater than 50 percent optical purity was obtained in this way.

E. Ylid Reagents - The same triphenylphosphine polymers 10 that were used for complexing metals can be utilized for the preparation of ylid reagents.[55-57] These reagents function in much the same way as their monomeric counterparts, but again provide for simpler, cleaner workups. In particular, the



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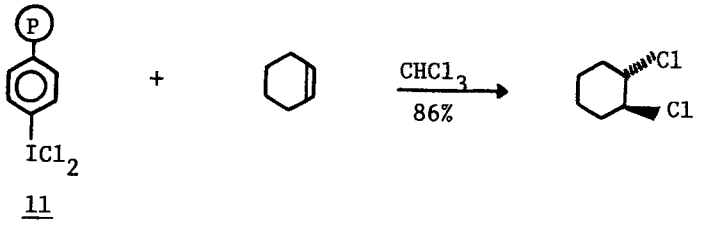
normal nuisance of removing triphenylphosphine oxide from the mixture is obviated.

F. Halogenation - Reagents have been prepared to carry out a variety of halogenation reactions. Included are (a) chlorination of olefins[58], utilizing the polymer 11, (b) conversion of acids to acid chlorides[59-61], (c) conversion of alcohols to chlorides[60,61] and (d) conversion of ethers to bromides.[62]



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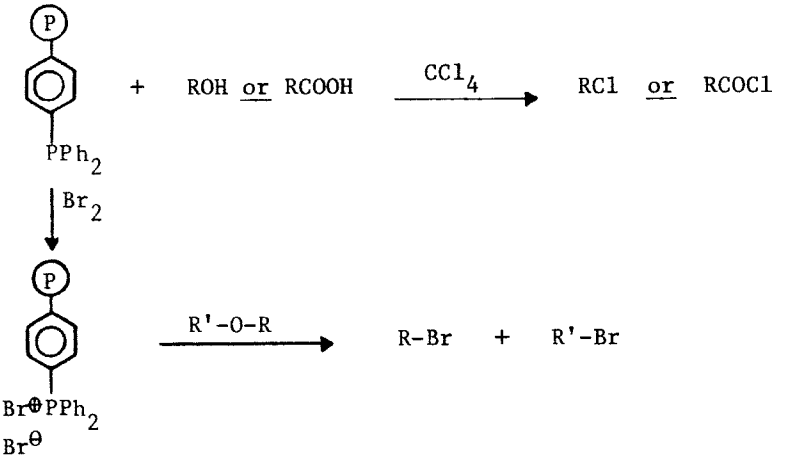
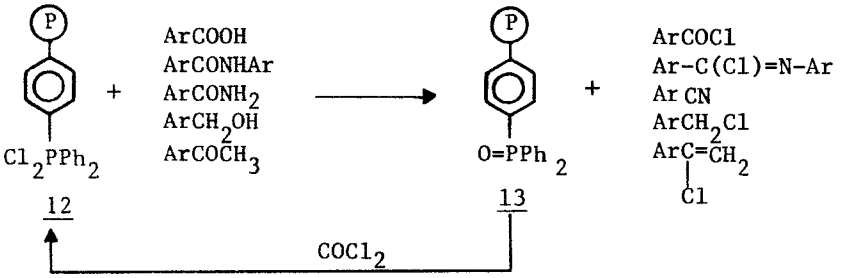
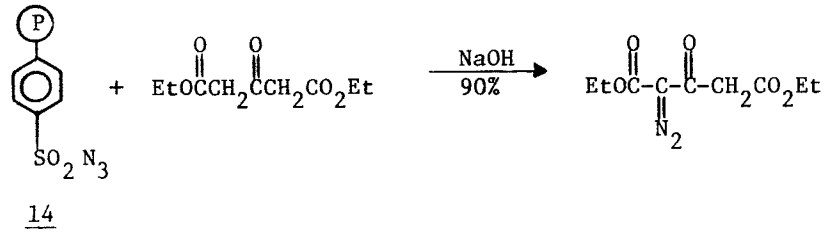


A polymeric triphenyl phosphine 12 dichloride is capable of performing a wide variety of reactions and the by-product phosphine oxide 13 can be easily recycled with phosgene.[60]

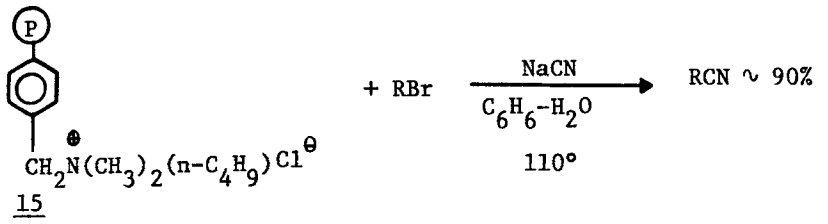


G. Miscellaneous - Rebek[63] has prepared the safe and stable polymer bound *tosyl azide* 14 and has utilized it in diazo transfer reactions with



activated methylene compounds.

A recent report describes the use of an insoluble polymeric catalyst 15 for the transfer of a nucleophilic species from an aqueous phase to an organic phase.[64] The method was found useful for assisting the reaction of hydrophobic alkyl halides with aqueous sodium cyanide.



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Aluminum chloride trapped within a polystyrene matrix has been reported to be stable to moisture and air, and to act as an efficient Lewis acid catalyst for the esterification of acids, formation of acetals from aldehydes and alcohols, and the preparation of labile ethers.[65-67]

A polymeric version of the useful coupling reagent "EEDQ" has been reported to effect the formation of peptide bonds between appropriately protected amino acids.[68] This material has the potential for use in automated processes and has been shown to be regenerative.

Polymeric carbodiimides such as 3 have also been used to form anhydrides from carboxylic acids[39] and preliminary experiments on peptide coupling have been performed.[69,70]

An acylating reagent [71,72] for alcohols and amines has been developed. A major advantage of this technique may be in the synthesis of radiolabeled compounds which are particularly relevant to biological studies.

H. Three Phase Test - Perhaps one of the most ingenious uses of these polymer supports has been demonstrated by Rebek[73-78] in what he calls the three phase test for reactive intermediates. Although this does not qualify as a reagent, the concept is so unique it is worth recounting here. Two different functionalized polymers are suspended in a common liquid. A reactive species is generated and set loose from one polymer and diffuses through solution into the second polymer where it is trapped by reaction with a polymer-bound substrate. By this method, Rebek has been able to prove the existence of uncomplexed cyclobutadiene, metaphosphate and the formation of an intermediate in the acylation reaction.

IV. Future - Promises and Problems - Our prognosis is that the future looks bright for polymeric reagents. It would have been an impossible task to predict the vast amounts of ion exchange resins in use today on the basis of early experiments conducted with these materials. We feel the general field of polymeric reagents is at just such an early stage. Monomeric reagents have been found that perform virtually any desired reaction necessary for synthesis, yet much of the time-consuming portion of the work continues to be product isolation and purification. From the preceding examples, it appears that virtually any type of reagent can be produced on a polymer given sufficient ingenuity, and that the advantages of simplified purification, ease of handling and increased safety will be of continuing importance.

But there are still significant problems in the field. Probably one of the most serious is standardization of the matrices by both the experimenters and the producers. A firmer understanding must be gained of the behavior of polymeric matrices in various solvent types, the molecular motion of the chains under different conditions and the effect of pore size. New methods of restricting the interaction of polymer-bound functional groups should be sought. The concept that lightly crosslinked materials are perfectly rigid and unflexible is just not acceptable. Even if we only partially substitute the rings, it is virtually impossible to know whether this has occurred in clusters or with random distribution; depending on the reagent, an argument could be made for either.[28] Certainly this would influence the local microenvironment and drastically modify reactions where one is looking for a dilution effect. Lastly, we would like to raise once again the issue of the limited methods of analysis of groups on the polymer. New methodology must be developed. This is particularly true if one wishes to do nonsequence synthesis on the polymer, an area that has only been touched on by Leznoff.[79]

In regard to medicinal chemistry, specifically, anything that helps one synthesize a new pharmaceutical more easily and in purer form should be of interest. We have tried to give a general overview of the subject with some specific examples, particularly when they relate to compounds such as prostaglandins. There are many more aspects to this subject that we could not cover in such a short review. Consider this a whetting of the appetite and utilize the many reviews cited in the references to further satiate any need for more information. Since this is the first time a chapter on this subject has appeared, we have chosen to cover more than one year's work.

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Chapter 30. The Chemical Modification of Cyclic AMP and Cyclic GMP.

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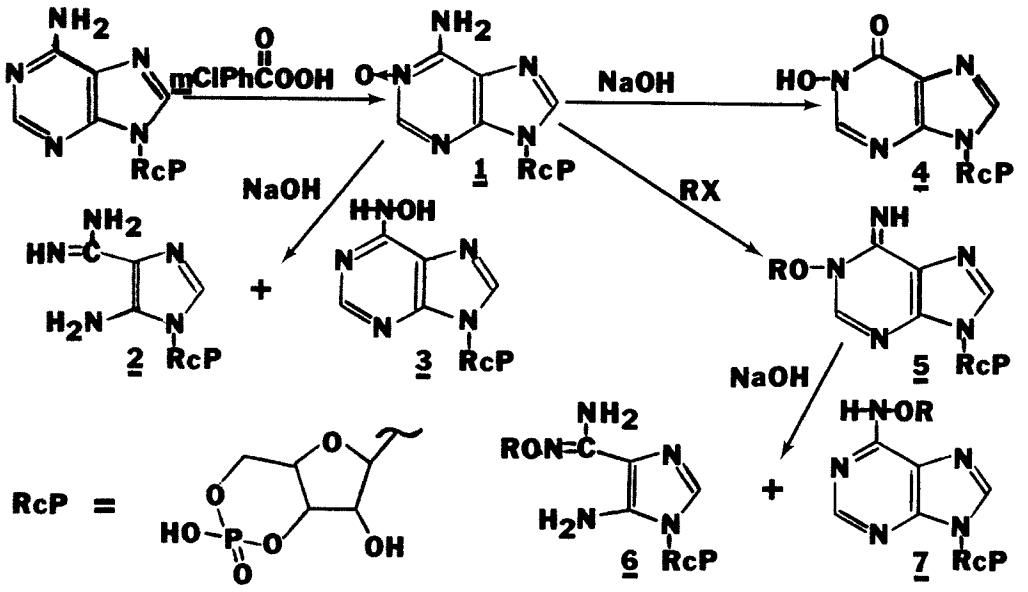
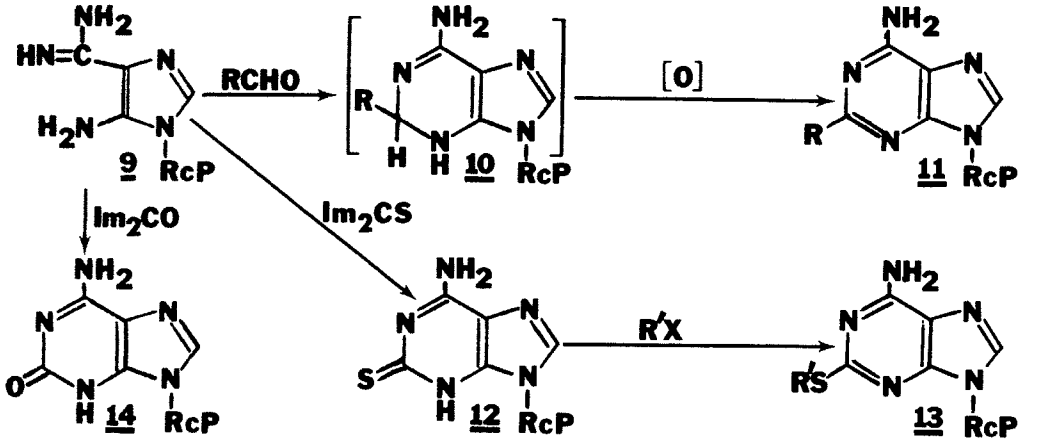
General. The relationship between the levels of cyclic nucleotides and various disease states is becoming better understood[1-3]. The primary mechanism by which cyclic nucleotides exert their biological effects is probably via the activation of cyclic nucleotide-dependent protein kinases. One way to alter the intracellular levels of cyclic nucleotides is by supplying derivatives which are potent protein kinase activators, which possess tissue specificity, and which are resistant to cyclic nucleotide phosphodiesterases. These latter enzymes hydrolyze the cyclic phosphate ring,and the resulting 5'-nucleotides are inactive. This review will summarize the more recently reported procedures for substitution or modification of cyclic phosphates in various positions of the purine ring. With some exceptions, modification of the ribose and cyclic phosphate moieties of cyclic nucleotides eliminates their biological activities[4], and therefore, these types of derivatives will not be discussed here. Previous reviews offer more detailed discussion of earlier work and the biological activity of the analogs[4-6]. Two general routes for the synthesis of analogs have been pursued. The first involves the formation of the 3',5'-cyclic phosphate ring from the corresponding nucleoside 5'-monophosphate. Earlier procedures to accomplish this[7-8] were inefficient and have yielded only a few cyclic nucleotide derivatives. A new cyclization method has recently been reported which appears promising[9]. The second general method, the direct substitution or modification of cAMP and cGMP has proven to be most productive and is the subject of this review.

N-1-Substituted Derivatives. cAMP N^1-oxide (1) was prepared by treatment of cAMP with m-chloroperbenzoic acid[10]. 1 with refluxing 2N NaOH gives 5-amino-1-β-D-ribofuranosylimidazole-4-carboxamidoxime cyclic 3',5'-phosphate (2), N^6-HO-cAMP (3), and cIMP N^1-oxide (4). 4 was also prepared by deamination of 1[10]. 1 with alkylhalides gave N^1-alkoxy-cAMP derivatives (5) which, upon treatment with dilute NaOH gave O-alkyl-5-amino-1-β-D-ribofuranosylimidazole-4-carboxamidoxime cyclic 3',5'-phosphates (6) and N^6-alkoxy-cAMP's (7)[10]. N^1-methyl-cAMP was synthesized by treatment of cAMP with MeI[10]. With the exception of 1, all of the above N^1-substituted (4, 5) and ring opened (2,6) cAMP derivatives were poor cAMP-dependent protein kinase (PK[cA]) activators. 3 and 7 demonstrated activity similar to that of cAMP with this enzyme. All of the above derivatives (1-7) were substrates for phosphodiesterase (PDE)[10].

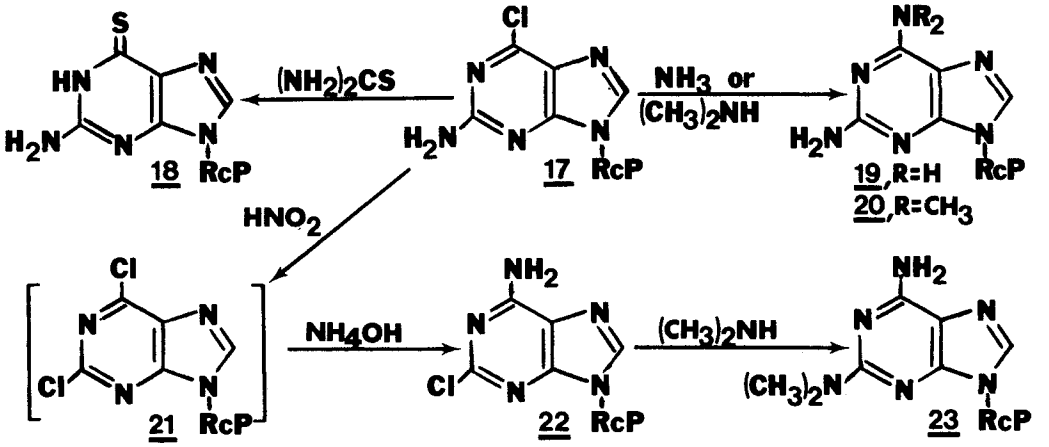
2-Substituted Derivatives. Reduction of 2 or its O-methyl derivative (6) with H_2 and Raney nickel catalyst gave 5-amino-1-β-D-ribofuranosylimidazole-4-carboxamidine cyclic 3',5'-phosphate (9)[10,11]. Treatment of 9 with the appropriate trialkyl orthoesters gave 2-methyl- and 2-ethyl-cAMP[11]. More generally, condensation of 9 with aldehydes under mild oxidative conditions led to conversion of the 2,3-dihydropurine intermediate (10) to a variety of 2-alkyl derivatives of cAMP (11)[11,12]. 9 with 1,1'-thiocarbonyl-



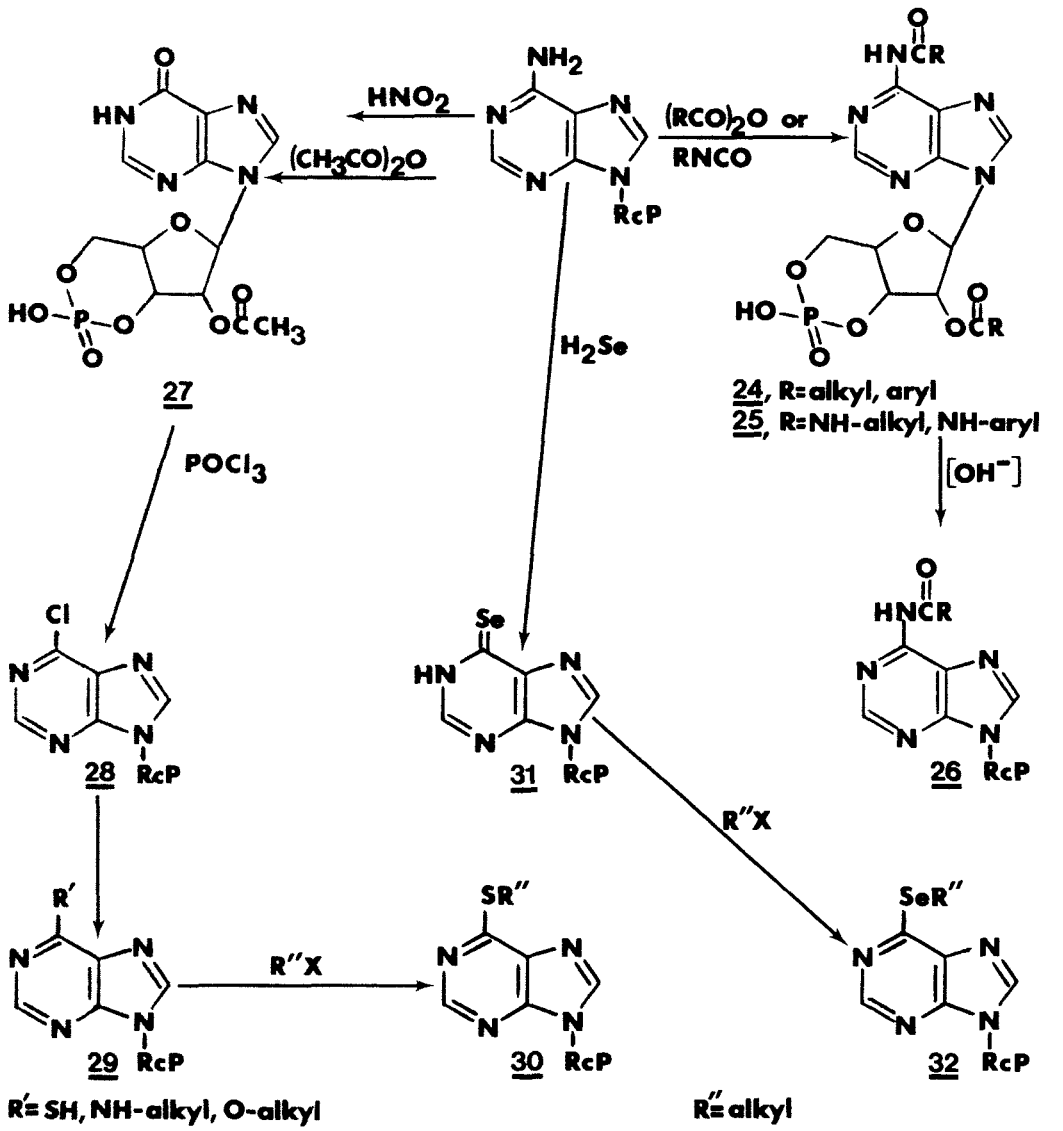
diimidazole gave 2-HS-cAMP (12) which upon treatment with various alkylhalides yielded 2-alkylthio-cAMP derivatives (13)[11,12]. 1,1'-Carbonyldiimidazole and 9 gave 2-HO-cAMP (14)[11], which has also been synthesized by the photochemical transformation of 1[13]. A few 2-alkyl- and 2-alkylthio-cIMP derivatives were prepared by deamination of the corresponding 2-substituted-cAMP derivatives (11, 13)[12]. An alternate route to the former is ring closure of 5-amino-β-D-ribofuranosylimidazole-4-carboxamide cyclic 3',5'-phosphate produced by the ring opening of cIMP[14]. Using multiple regression analysis, a striking relationship was found between the relative potency of various 2-substituted cAMP derivatives as PK[cA] activators and parameters describing the hydrophobic, steric, and electronic character of the substituents (r=0.967)[12]. These derivatives (11-14) were good substrates for PDE[12,14]. Some 2,6-disubstituted derivatives



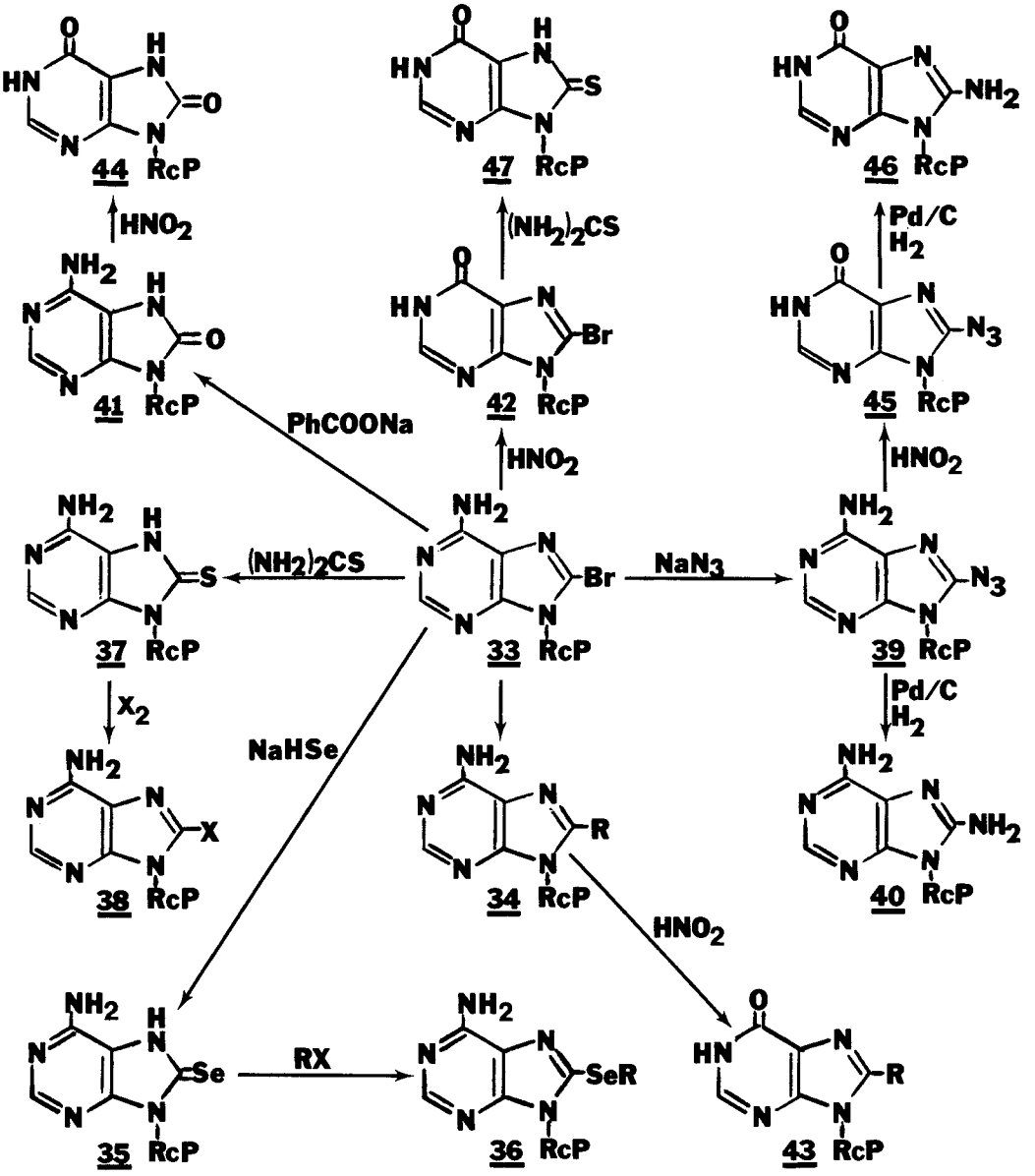
of 9-β-D-ribofuranosylpurine cyclic 3',5'-phosphate (nebularine cyclic 3', 5'-phosphate, cNMP) have been synthesized from cGMP[15]. The intermediate 2-H_2N-6-Cl-cNMP (17) was provided by treatment of 2'-O-acetyl-cGMP with phosphoryl chloride-N,N-diethylaniline. Treatment of 17 with the requisite nucleophile gave 2-H_2N-cNMP-6-thione (18), 2-H_2N-cAMP (19) and 2-H_2N-N^6,N^6-dimethyl-cAMP (20). Treatment of 17 with HNO_2 and conc. HCl gave 2,6-Cl_2-cNMP (21), which was converted *in situ* to 2-Cl-cAMP (21) with conc. NH_4OH. 22 was also obtained from treatment of 12 with Cl_2[15]. Nucleophilic substitution of 22 yielded 2-$(CH_3)_2N$-cAMP (23). Oxidation of 21 in a manner analogous to the synthesis of 1 gave 2-Cl-cAMP N^1-oxide. Results with these derivatives (17-22), as well as 12, 14, and xanthosine cyclic 3',5'-phosphate (cXMP) indicated that cGMP-dependent protein kinase (PK[cG]) is highly specific for cyclic nucleotides containing an amino group in the 2-position, while PK[cA] is specific for those containing an amino group in the 6-position[15].



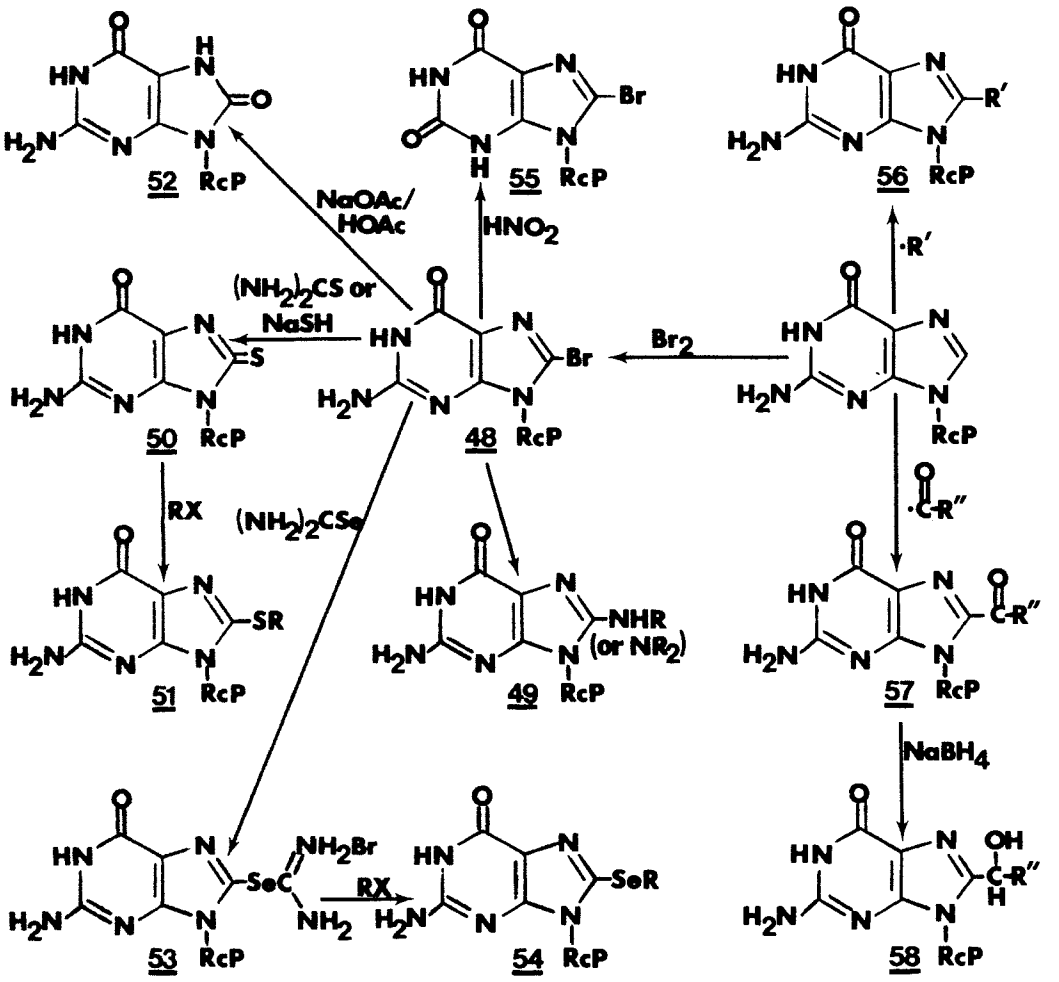
N^6-and 6-Substituted Derivatives. N^6-acyl- and N^6-carbamoyl-cAMP derivatives were prepared by treatment of cAMP with acid anhydrides[16] or N-alkyl (or acyl) isocyanates[17] to yield the N^6,2'-O-bis(acyl)-cAMP's (24) and N^6, 2'-O-bis(N-substituted-carbamoyl)-cAMP's (25). Selective hydrolysis of 24 and 25 gave the N^6-acyl- and N^6-carbamoyl-cAMP derivatives (26)[16,17]. These compounds demonstrate the same order of activity as cAMP with PK[cA] and are generally resistant to PDE[17,18], but have the drawback that some of them are probably enzymatically deacylated. More stable N^6- and 6-substituted derivatives have been synthesized[14,19]. cAMP was deaminated and the resulting cIMP was acetylated (27) with acetic anhydride prior to chlorination in refluxing $POCl_3$ to yield 6-Cl-cNMP (28). Treatment of 28 with nucleophiles gave 6-SH, 6-alkylamino-, and 6-alkoxy-cNMP derivatives (29). 6-SH-cNMP was converted to 6-alkylthio-cNMP derivatives (30) by reaction with alkylhalides[19]. cAMP was converted to 6-selenoxo-cNMP (31) by treatment with H_2Se in aqueous pyridine[20]. 31 was transformed to 6-alkylseleno-cNMP derivatives (32) with alkylhalides. Many of these N^6- and 6-substituted derivatives (29,30) were able to activate both PK[cA] and PK[cG], but were also good substrates for PDE[14,19].



8-Substituted Derivatives. 8-Br-cAMP (33) was prepared by direct bromination of cAMP[21]. Nucleophilic substitution of 33 gave 8-alkylamino-, 8-alkylthio-, and 8-alkoxyderivatives (34)[21,22]. NaHSe and 33 led to 8-seleno-cAMP (35) which with alkylhalides yielded 8-alkylseleno-cAMP's (36)[23]. Thiourea treatment and 33 gave 8-HS-cAMP, which was converted to other 8-halo-cAMP's (38)[24]. Azide yielded 8-N_3-cAMP (39), which was hydrogenated to give 8-H_2N-cAMP (40). 8-HO-cAMP (41) can be obtained from 33 by treatment with Na benzoylate[14]. 8-Substituted cIMP derivatives were prepared by deamination of the corresponding 8-substituted cAMP derivatives: 8-Br (42)-, 8-alkylthio(43)-, 8-HO (44)-, and 8-N_3 (45)-cIMP[22]. 42 underwent nucleo-

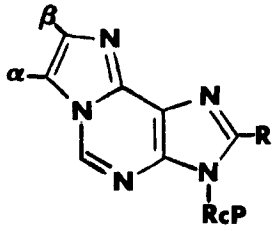


philic displacement to yield 8-alkylthio-, 8-alkylamino-, and 8-alkoxy-cIMP derivatives[14]. 8-H_2N-cIMP (46) came from reduction of 45, and 8-HS-cIMP (47) from thiourea treatment of 42[22]. 8-Br-cGMP (48) and nitrogen nucleophiles gave 8-alkylamino-cGMP's (49)[14,22]. Thiourea or NaSH and 48 gave 8-HS-cGMP (50) which was converted to 8-alkylthio-cGMP derivatives (51) with alkylhalides[14,22]. Refluxing 48 in NaOAc-glacial HOAc gave 8-HO-cGMP (52). 48 and selenourea gave the 8-isoselenouronium (HBr)-cGMP (53) which



was treated with alkylhalides to yield 8-alkylseleno-cGMP derivatives (54)[25] Deamination of 48 gave 8-Br-cXMP (55)[22]. cGMP underwent homolytic alkylation and acylation to yield 8-alkyl(56)- and 8-acyl(57)-cGMP derivatives[26]. The latter (57) were reduced to the corresponding 8-(1-hydroxyalkyl)-cGMP derivatives (58)[26]. 8-substituted-cAMP derivatives were specific for PK[cA], while 8-substituted-cGMP derivatives were specific for PK[cG][22]. 8-substituted-cIMP derivatives stimulated both kinases with some preference for PK[cA][22]. The general order of activity is the same for the three groups of derivatives: -Br, -OH, -SH, -SR> $-NH_2$, N_3>NR[21,22]. With PK[cG] the 8-acyl- and 8-alkyl-cGMP derivatives demonstrated the following order of activity: 1-hydroxy-alkyl>acyl>alkyl[26]. With the exception of the 8-NH_2 derivatives the 8-substituted cyclic nucleosides are quite resistant to PDE[14,21-26].

1,N^6-Etheno Derivatives. Chloroacetaldehyde and cAMP gave 3-β-D-ribofuranosylimidazo[2,1-i]purine cyclic 3',5'-phosphate (59, 1,N^6-ethano-cAMP)[27]. Similar reactions starting with 8-Br-, 8-CH_3S-, and 8-$PhCH_2S$-cAMP led to the corresponding 8-substituted-1, N^6-ethano-cAMP derivatives (60)[28]. Condensation of cAMP with α-bromoacetophenone gave the β-phenyl-derivative of 59 (60). On the other hand, treatment of cAMP or 8-CH_3S-cAMP with 2-bromovaleraldehyde or treatment of cAMP with 2-bromo-2-phenylacetaldehyde yielded the corresponding α-substituted derivatives: 62 and 63[28].



59, R = -H, α = -H, β = -H

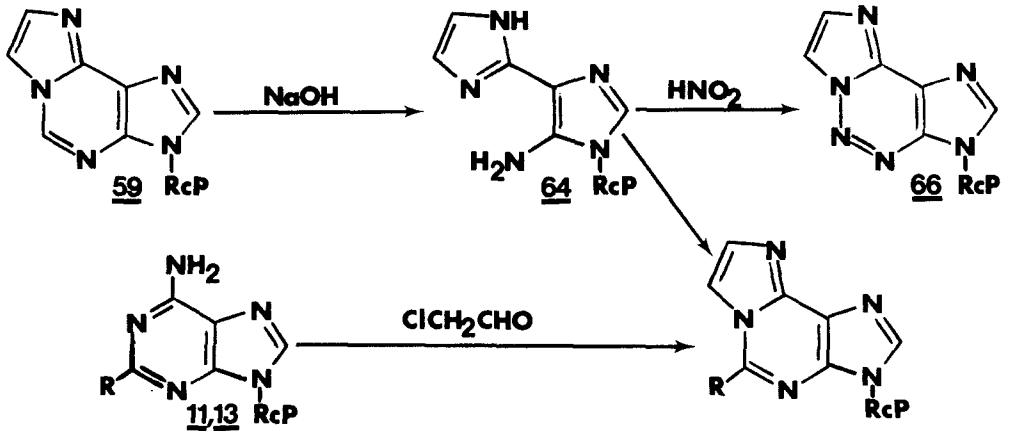
60, R = -Br, $-SCH_3$, $-SCH_2Ph$, α = -H, β = -H

61, R = -H, α = -H, β = -Ph

62, R = -H, α = -n-C_3H_7, -Ph, β = -H

63, R = $-SCH_3$, α = -n-C_3H_7, β = -H

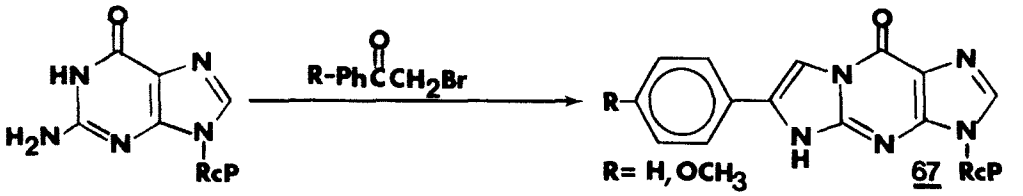
The general order of activity of these etheno-cAMP derivatives with PK[cA] is 63≈61>60>59≈cAMP>58[28], and 59 and 61 were hydrolyzed at 10 and 20% the rate of cAMP, respectively[10]. The synthesis of 2-substituted derivatives of 59 was approached by reaction of 2-substituted cAMP derivatives (11, 13) with chloroacetaldehyde, or by ring opening of 59 in alkali to give 5-amino-4-(imidazo-2-yl)-1-β-D-ribofuranosylimidazole cyclic 3',5'-phosphate (64)[29,30] with subsequent ring closure like that performed with 9 to 2-substituted 1,N^6-etheno-cAMP derivatives (64)[30]. Treatment of 64 with HNO_2 gave 2-aza-1,N^6-etheno-cAMP (66)[29,30].



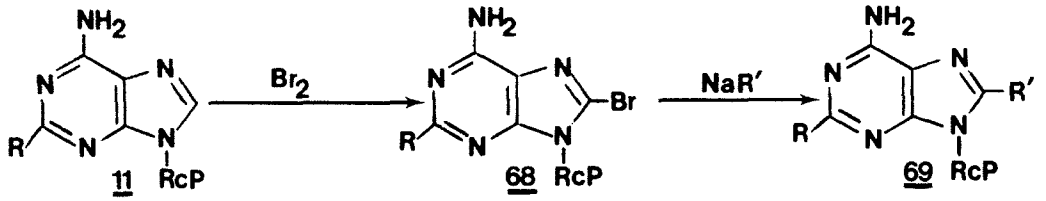
Compounds of the type 65 fell into two groups as PK[cA] activators: Those where the 2-substituted cAMP derivative (11, 13) was more active than the corresponding ethano derivative (R = H, $-CH_3$, -n-C_4H_9; and $-SC_2H_5$), and

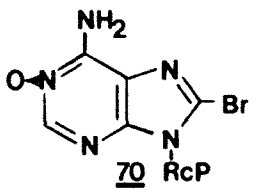
those where the etheno derivative was more active (R = -Ph, 2-aza, and 64 vs 9)[30]. The etheno substitution substantially decreased the ability of the 2-substituted cAMP's to serve as substrates for PK[30].

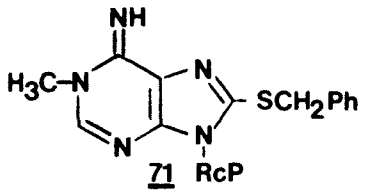
1,N^2-Etheno Derivatives. Treatment of cGMP with phenacylbromide or p-methoxyphenacylbromide gave the corresponding 1,N^2-etheno-cGMP derivatives (67)[30], which were 1/6 to 1/3 as active as cGMP with PK[cG]. Interestingly, they are also 1/10 to 1/5 as active as cAMP with PK[cA][30]. They are only slowly hydrolyzed by PDE[30].



1,8- and 2,8-Disubstituted Derivatives. 2-CH_3- or 2-nC_4H_9-cAMP were brominated in a manner analogous to the preparation 33, and these 8-Br derivatives (68) were subjected to nucleophilic displacement in a manner similar to that of 33 to yield the 2,8-disubstituted-cAMP derivatives (69)[31]. 33 was oxidized as was cAMP 23 to give 8-Br-cAMP N^1-oxide (70)[31] and 8-$PhCH_2S$-cAMP was methylated as was cAMP[10] to yield N^1-CH_3-9-$PhCH_2S$-cAMP (71)[31]. Depending on the substitutions, examples were found where the disubstituted derivatives were either more active, equally as active, or less active than the monosubstituted parent compounds as PK[cA] activators [31]. 8-substitution completely or substantially eliminated the ability of 1- or 2-substituted derivatives (1, 8, 11, 13) to serve as PDE substrates[31].

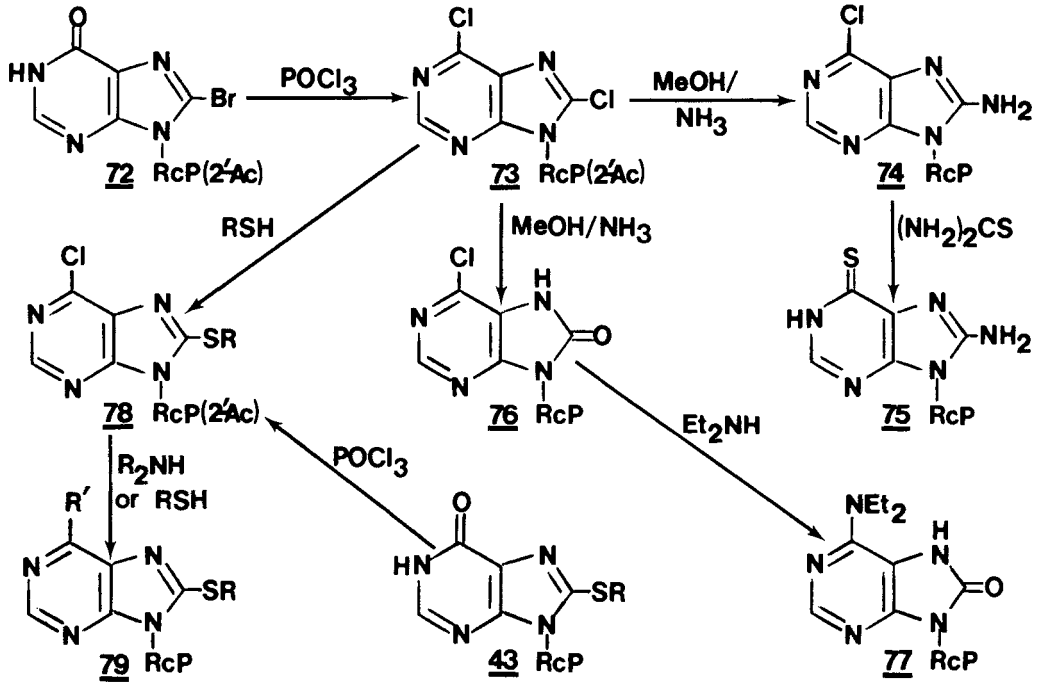






6,8-Disubstituted Derivatives. Acetylation of 42 led to 2'-O-acetyl-8-Br-cIMP (72) which when refluxed with phosphorus oxychloride gave 2'-O-Ac-6,8-Cl_2-cNMP (73)[31]. Treatment of 73 with MeOH/NH_3 gave 8-NH_2-6-Cl-cNMP (74), which was converted to 8-NH_2-6-HS-cNMP (75) with thiourea; and also gave

6-Cl-8-HO-cNMP (76), which was converted to N^6-Et_2-8-HO-cAMP (77) with Et_2NH. 8-alkylthio-6-substituted cNMP derivatives (79) were formed by nucleophilic displacement of the 8-Cl of 73 to give 2'-O-acetyl-8-alkylthio-6-Cl-c-NMP (78), which was subjected to attack by alkylthio and alkylamino nucleophiles to give 79. The latter were also produced by treatment of 43 with phosphorous oxychloride to yield 78 which were converted to 79 in a similar manner as above[32]. In general these derivatives (74-78) were intermediate between the N^6- (or 6-) and 8-substituted parent compounds in activity with PK[cA] and completely resistant to PDE[33].



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Chapter 31. Quantitative Drug Design

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"Quantitative drug design" embraces two major activities; the quantitative description of the structural differences among series of chemical compounds of biological interest, and the formulation of "quantitative structure-activity relationships" (QSAR) useful in the design of new and better therapeutic agents. The following review of advances in the last three years[1] is organized to parallel this definition (in contrast to many of the recent general reviews[2-8] which partition the subject into methods such as "Hansch" or "Free-Wilson"). A discussion of progress in the various numerical descriptions of chemical structure will be followed by the advances in methods for extracting the QSAR's themselves. A final section highlights some noteworthy results.

Structural Descriptors. A drug design problem begins with a series of more-or-less related chemical structures, each associated with differing biological observations. Obviously it cannot be the differences among the structural diagrams which themselves produce the biological differences! The latter can result only from differences in physical properties, resulting in altered transport, metabolism, or receptor binding. Therefore the drug designer will usually translate the structural diagram differences into a series of physical property differences that could plausibly be relevant to biological behavior. The translation can be done by actual laboratory measurement, if the compounds are available, or more often by calculations of various degrees of complexity. A sensible strategy[9-10] is to try the most convenient translation first and add complexity if required.

However, one should first consider the vexing question of which species (protomeric, tautomeric) is involved in the biological system.[11] Indeed, both tautomers of the imidazole ring may be required for agonism of the histamine H_2 receptor,[12] whereas the antibacterial form of kojic acid is neutral.[13]

Substituent Contribution Model. Often the series of compounds varies only in the nature of simple substituents, a nucleus or "lead structure" being constant. The effects of such substituent differences on the molecule's polarity, lipophilicity, and bulk will be similar from series to series, and so the translation of structural diagrams into physical properties can be a simple matter of copying appropriate substituent parameters from one of several recent compilations.[14-16] Multiple substitution is commonly handled by summing the parameters of individual substituents.[17]

The polar effects of substituents are usually expressed satisfactorily by the classical Hammett δ,[18] although the combination of F (inductive) and R (resonance) effects gave a better account of phenol toxicity to the

algae Daphne.[19] An interesting development is the observation by several groups[20-23] of superior potency correlations using sp^2 oxygen IR stretching frequencies. A quantum statistical argument,[24] that differences in IR stretching frequency may in fact cause differences in receptor site binding, seems to be supported by the observation that odor quality is a complex function of far infrared frequencies.[25] The merits of a proposed transfer constant C_T are difficult to appreciate.[26-27]

Lipophilicity is the most frequent determinant of biological potency, as evidenced by the compendia of QSAR involving partition coefficients.[28-29] The calculation of partition coefficients from structure by summing contributions of individual fragments has been extended and improved.[30-31] Expression of the partition coefficient of a molecule in terms of a balance between large volume (hydrophobic forces) and polar groupings (hydrophilic forces) has been utilized successfully in calculation of π[32] and R_M values.[33] Appropriate "hydrophilic constants" have been derived from the partitioning of various solutes between the vapor and aqueous solution phases.[34]

Techniques for measurement of partition coefficients are also much improved. Physical data for a variety of candidate solvent pairs indicate that n-octanol is a superior biological membrane model.[35] Inclusion of a hydrogen-bonding parameter allows more accurate interconversion of data from different solvent systems.[36] A method is described for simultaneous determination of pK_A and partition coefficient.[37] Although the partition coefficients of individual ionic species are less variable, overall distribution coefficients for drugs with pK_A's near physiological pH can show a pronounced temperature dependence.[38] Measurement of reversed phase TLC values[39] can be more convenient experimentally than "classical" partition coefficient determination, although ionizable compounds present problems.[40] Polyamide TLC[41] seems less satisfactory.[42] There is enthusiasm for the simplicity and accuracy of partition coefficient determination by HPLC on octadecasilyl columns,[43-44] which may be most satisfactory for non-basic compounds.[45] Although measured partition coefficients are naturally preferred over calculated values,[31] the few studies which would be expected to support this preference are inconclusive.[46-47]

While most workers view correlations with π in terms of overall distribution properties, reports[48-50] of "directional hydrophobicity", i.e., series in which a π dependency exists only for variation at one among several substituent positions, are interpreted as evidence for the heterogenous character of enzyme binding sites.[51]

As would be expected, steric effects of substituent variations have proved most difficult to quantify. Polarizability (MR) was once recommended[17] as a generalized measure of bulk, but its interpretation is now less clear.[51] The leading alternatives are the E_S parameter and its elaborations,[52] molecular volume,[53] and parachor.[54] Two parameterizations which give greater weight to substituent fragments distant from the point of attachment are the Newman "six-number",[55] and the ratio of β group volume to α group volume.[56] A noteworthy development is a branching

parameter derived from graph theory, which is a predictor of R_M values and boiling points.[57] As an indication of the interest in better steric parameters, within a week QSAR appeared involving this new branching parameter, newly baptised as $^1\chi$![58] Comparisons with other parameters[58A] suggest that $^1\chi$ indeed measures the volume of a substituent composed of first-row elements.

It is mildly surprising that a generalized hydrogen-bonding parameter has yet to be developed. Hydrogen-bonding contributes to anesthetic potencies,[59-61] and a correlation involving the stretching frequency of an intramolecular hydrogen bond between a series of alkoxy groups and an adjacent N-H has been reported.[62] Another source of substituent parameters, quantum based calculation,[63] is seldom employed lately. Presumably, the parameter tabulations have proven more useful in practice.

It is possible that familiar parameter scales such as π are in reality experimentally convenient approximations to thermodynamically better defined concepts such as activity coefficients.[64] For example, an improved account of anesthetic potencies is obtained by the use of activity coefficients, molar volume, and a hydrogen-bond scale,[61] rather than π and a substructural variable for hydrogen bonding,[60] although the latter study encompasses more structural diversity.

Larger substituents having greater conformational flexibility are less likely to exert simple additive hydrophobic, electronic, and steric effects. Nevertheless a large set of such data may still be dramatically simplified[65] by defining elementary parameters for some or all of the complex substituents present (= 1 for a molecule having the specified substituent and = 0 otherwise). Such binary parameters are styled "substructural", "indicator", or "de novo" variables. (The Free-Wilson method[66-69] is the limiting example of this approach, in that all substituent/position combinations are expressed as substructural variables and the objective of the analysis is to determine whether substituent effects are additive). Furthermore the recent instances of studies involving large numbers of compounds falling into no coherent series are all based on substructural variables. With no single "lead structural nucleus" to establish the boundaries of a "substituent", the substructures in these studies have usually been drawn from the units of a preexisting structure retrieval system,[70] most often based on a mixture of ring systems and the more elemental augmented atoms (i.e., groups composed of atoms with their nearest neighbors).[71-73]

There is certainly no theoretical objection to mixing parameter types, and some exemplary studies have combined substructural and physicochemical parameters[74-75] or empirically-based partitioning and quantum calculated parameters.[76]

Molecular Shape. Advances are needed in the methodologies for quantitative description of the overall molecule. Two difficulties are involved. The first is the description of shape itself, or "conformational analysis". The second difficulty, often not even articulated, is the characterization

of shape in a fashion that can be usefully related to potency. (For example, knowledge of the rigid shapes of many morphine-like analgesics has been of little help in formulating SAR.)

The greatest contributions of quantum mechanics to quantitative drug design continue to be in the calculation of conformations. From excellent recent reviews[77-78] and symposia proceedings,[79] it would appear that cost and methodological uncertainties still require quantum calculations of conformation to be a "one-molecule-at-a-time" process, limited in practice to biogenic molecules of broad academic interest, e.g., a detailed study of phenethylamine.[80] Nevertheless steady improvement in computing technology is allowing greater sophistication in the formulation of conformational problems. For example, aqueous solvation is found to reduce the energy differences between conformations, using either a "supermolecule" model, in which explicit solvent molecules are allowed to promenade about the solute as the system energy is minimized,[81] or various continuum models.[82] Larger molecules can be handled by a "molecular decomposition" technique,[83] first introduced to permit ab initio calculations[84] on molecules as large as lincomycin and recently used with the less demanding CNDO method on disaccharides.[85] Whenever calculation of entire potential energy surfaces is feasible, the probability as well as the energy content of individual conformations can be determined and thus overall conformational equilibria delineated.[86-87] The value of such a larger effort is illustrated by the finding that the conformer of histamine which appears to stimulate the H_1 receptor represents a local maximum.[88] Moreover, the population of such high energy conformations will be underestimated if bond distances are not allowed to deform as a bond angle is rotated.[89]

In view of the importance of shape to biological activity on the one hand and the cumbersome nature of the experimental and quantum methods for determining shape on the other hand, it is surprising that so little attention has been paid to conformational calculations using empirical potential functions, such as Van der Waals forces and electrostatic interactions.[86,90] These much less expensive methods successfully reproduce the existing experimental data for molecules as varied as acetylcholines,[91] phenethylamines,[92] disaccharides,[85] and polypeptides.[86]

Approaches to the characterization of shapes in a form useful for QSAR remain preliminary. Receptor-mapping, in a simplistic formulation which ascribed high probability to activity to structures containing a triangle of specific size defined by atoms of specified type,[93] produced histamine and muscarinic "pharmacophores," among others, which have not been confirmed experimentally.[94,95] A more sophisticated form of receptor mapping involves construction of an electrostatic potential map, which describes the force encountered by a charged particle at any point about a drug molecule.[96-99] For example, a comparison of such maps for isoproterenol and INPEA suggests that an unexpectedly high electric field about the phenyl ring distinguishes β agonist from antagonist activity.[100] A related idea is the "mean steric difference" (MSD).[8,101-103] Surprisingly good correlations are obtained for enzymatic hydrolysis rates when the structural differences of a substrate from some standard are expressed only

as the number of non-hydrogen atomic replacements that would be necessary to convert the substrate to the standard. Superposition of molecular silhouettes is a similar approach.[104-105] It has been observed[106] that toxicity is more frequent in symmetric than unsymmetric compounds.

Graphical display systems for the computer manipulation of three-dimensional information[107] are a tool often advocated for further characterizing pharmacophores. Yet no experienced medicinal chemist will place much faith in any SAR derived from at most a half-dozen structures, and thus the real requirement for progress in the elucidation of pharmacophores is a rapid and dependable conformational calculation method. Incidentally, the overhead costs of occasional large-scale computer calculations are high enough to suggest that industrial customers would exist for well-documented quantum, conformational, or pattern-recognition programs, accessible on a system such as Prophet[108] or the National Resource for Computation in Chemistry.[109]

The Derivation of QSAR's. Having by some of the above techniques obtained various columns of commensurable structurally-dependent parameters, the drug designer must now discover which, if any, of these columns is related to potency. These tasks deserve equal attention. All too many studies combine sophisticated data acquisition with naive data analysis (or the reverse).

If there is convincing evidence that potency must depend on at most two structurally-related variables, then plots will be a satisfactory method for establishing the SAR. Furthermore, graphically based strategies such as the Fibonacci[110-111] and Simplex techniques[112] should efficiently identify any optima. However, when approaching a new series of data, it is very difficult to be sufficiently confident that any particular variable or variable pair is uniquely relevant. The "Topliss tree"[113] and cluster analysis-based methods of selecting substituents[17,114] consider a greater range of possible substituent effects. Synthesis must after all start somewhere, and the latter methods maximize the chances of early identification of a useful QSAR.

However for problems involving biological data of doubtful precision, large numbers of possible SAR, and even larger numbers of compounds - in other words, most drug design problems - regression or correlation analysis has achieved a well-deserved popularity. (Indeed this reviewer suspects that within ten years its use will be a requisite for publication of any manuscript describing a series of more than a dozen compounds!) The techniques and pitfalls of regression analysis have been well described.[115] The danger of obtaining chance correlations by working with too few observations (compounds)[116] is widely known. Less appreciated is the possibility that many statistically acceptable correlations can be hidden within any particular set of data, as illustrated by successive studies[117-119] of progestational steroids. In such a situation, until more compounds are synthesized, designation of a particular equation as "best" is primarily a matter of taste, for which some guidelines[120] have

been proposed. Discriminant analysis is a technique similar to regression analysis that has been used to classify MAO inhibitors.[121]

Although linear relationships between potency and the structural variables are the most likely a priori, the simplest to validate, and usually have the most valuable synthetic implications, there often may be a question of which type of non-linear relationship is most reasonable to seek. For example, alternatives to the familiar parabolic dependence of potency on π have been proposed on theoretical grounds.[122-123]

The initial enthusiasm[124-126] for pattern recognition methods as drug design tools has been tempered by increasing appreciation of the practical difficulties.[127-130] Specific methods such as the learning machine[131] can indeed develop criteria for widely diverse structures which, to a limited degree, separate active compounds from inactive compounds,[72,132] often in a predictive[70-71] as well as a retrospective sense. Comparative studies[133] suggest that the simplest of these methods perform equally well. In the most impressive demonstration of pattern recognition techniques in drug design,[134] multi-dimensional scaling was used to reduce a variety of subjective perceptions of odor of a hundred compounds into a two-dimensional pattern of points or "odor spectrum". Then an economical description of the odor spectrum in terms of physicochemical, substructural, and spectral descriptors was sought using a variety of pattern recognition techniques. The results are more consistent with the IR absorption theory[25] of odor perception than with the shape-fitting theory.[105] This study suggests that pattern recognition will be particularly useful when the design objective is a complicated profile of biological activities, rather than simply a maximized potency and minimized toxicity(s). A further example is the use of factor analysis to reduce the number of observations needed to characterize a CNS-active drug.[135]

Biological Data. Usually biological activity is expressed as the logarithm of the dose necessary to produce a standard response, that is, in units of free energy. However, successful correlations have also been obtained using response at a standard dose[136] or its logit transform.[69] Also it may be highly desirable to include "inactive" compounds in an analysis,[137] despite a cost in precision, by assigning to inactives an arbitrary potency substantially below the lowest potency that the biological test protocol seems capable of observing.[68-69]

Drug Design Results. Without doubt, the results most demanded of drug designers are successful predictions of compounds that are more potent than their predecessors. Indeed, the past three years have seen a steady increase in the number and quality of such predictions. A first group of predictions[75-76, 138-141] produced slight increases in potency (<.5 log units), usually resulting from replacement or rearrangement of the simpler aromatic substituents. Encouraged by these modest triumphs, drug designers have recently been recommending more substantial structural alterations. It may be significant that the most promising reports of successful prediction are emerging from cancer research, which enjoys more rapid turnaround and less stringent proprietary considerations than

most drug research. Activity against brain tumors was obtained from alkylating agents by attaching rings of sufficiently high hydrophobicity.[142-143] And, following earlier analysis,[144] greater potency and lower toxicity are obtained from the well known anti-neoplastic agent CCNU by replacing its cyclohexyl group with a carbohydrate moiety.[145] Thus,it is not surprising that modification of the anticancer screening systems to conform better with considerations of hydrophobicity is being seriously discussed.[146] However,an ad hoc IUPAC committee has recommended a ban on the publication of experimentally untested potency predictions, fearing an adverse effect on the profit motivation for verifying the prediction.[147]

The potential of drug design for greater future contributions is also apparent from the increasing scope of recent studies. The current "records" for sheer size appear to be 256 compounds for studies involving regression analysis[74] and 1260 compounds overall.[70] Among the instances of QSAR having more general applicability than might at first have been expected are: the MAO-inhibitory structures phenylhydrazines, α-methylbenzylamines, carbolines, pargylines, and phenylcyclopropylamines all exhibit similar QSAR;[148] replacement of COO^- by $C\equiv C\text{-}\Delta\text{-}COO^-$ does not alter fibrinolytic SAR;[149] and in vitro/in vivo correlations exist involving microsomal oxidation with cholesterol biosynthesis[150,151] and neuronal membrane potential with analgesic[152] and CNS depressant actions.[153] A frequently sought pharmaceutical objective, achievement of oral activity, may be facilitated by the findings[154-156] that, while gastric absorption increases continually with lipophilicity, an optimum partition coefficient exists for intestinal absorption, whose logarithm is unexpectedly low, between 1.0 and 2.0. Optimal partition coefficients are proposed for a variety of other transport processes.[157] Finally, the hallucinogenic potency of amphetamines has been correlated with lipophilicity,[158] providing a first QSAR in the target species itself - man!

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Chapter 32. Magnetic Resonance Probes of Drug Binding

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N.m.r. has found widespread application in biological chemistry not only in its traditional role as a tool for molecular structure determination, but increasingly as a spectroscopic probe of labile associations between macromolecules. Noncovalent complexes are of particular relevance to problems of drug and hormone action and this report reviews recent applications of magnetic resonance in this field. Pharmacological[1-3] and biochemical[4,5] applications of n.m.r. were last reviewed in 1973. Paramagnetic ion probes of enzyme function[6], and n.m.r. studies of RNAase complexes[7] and DNA-histone complexes[8] have also been reviewed.

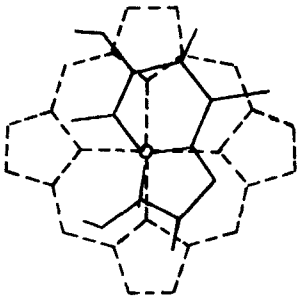
Complexes of ATP with Neurotransmitters - Various neurotransmitters form labile complexes with ATP that can be observed by n.m.r. The fact that synaptic vesicles and chromaffin granules contain both neurotransmitter and ATP in high concentration suggests that these complexes may be relevant to neurotransmitter storage. Complexes of catecholamines[9,10] and acetylcholine[11,12] are bound primarily by ionic forces and exhibit a stoichiometry of 3:1 or 4:1 (amine:ATP), depending on pH. Serotonin forms a high molecular weight micellar complex with ATP at a stoichiometry nearer 2.0-2.5:1[13], and also forms aqueous complexes with pyrimidine nucleotides[14]. The micellar complex, which is of possible physiological significance in serotonin storage vesicles in platelets and brain, shows substantial upfield chemical shifts in the aromatic protons of serotonin and in H-2 on the purine ring of ATP. Binding is attributed partially to base stacking, which explains the observed upfield shifts through the shielding effects of ring currents, and partially to ionic interactions, which result in relative immobilization of the polar sidechain and concomitant broadening of the side-chain proton resonances. The concentration dependence of the indole proton chemical shifts are consistent with an association constant of $K=6.2\ M^{-1}$.

Acetylcholine-ATP complex formation is accompanied by little if any change in proton shifts but can be detected by the shortening of relaxation times (T_1) of the acetylcholine protons[11]. Addition of ATP to a limiting stoichiometry of 1 ATP:4 acetylcholines shortens T_1's of all the protons by the same fractional amount indicating that both ends of the molecule are immobilized equally. In all these complexes, chemical exchange is very rapid on the n.m.r. time scale. Hence, the observed relaxation rate (or chemical shift) is a weighted average of relaxation rates (chemical shifts) in bound and free sites.

Ternary Complexes Containing Metal Ions - Acetylcholine also forms a complex with Mn(II)-ATP[12]. Ternary complexation has been inferred from the fact that paramagnetic broadening due to Mn(II) of the acetylcholine occurs only when ATP is present. The binary complex appears to be stronger than the ternary complex however (as expected on electrostatic grounds)

and is more likely of physiological significance in synaptic vesicles[15]. Rather surprisingly, ternary complex formation of spermine to Cu(II) and AMP did not noticeably affect either the ^{13}C or ^{31}P spectra[16]. Complex formation was inferred from a large change in the Cotton effect of AMP in ternary relative to binary solutions. Binding of Cu(II) to the N-7 position of AMP is clearly apparent in the n.m.r. spectra, and involvement of the ribose hydroxyls in the binding of spermine has been suggested to reconcile the ORD data with the small Cu(II)-spermine interaction observed in the n.m.r. spectra. ADP, Mn(II) ion and nicotinamide form a ternary complex that is observable from broadening due to Mn(II) in the proton spectra[17] of nicotinamide and appears to involve hydrophobic stacking interactions between adenine and nicotinamide rings.

Structures Derived from Pseudocontact Shifts - A very detailed picture of base-stacked complexes of caffeine and metal porphyrins has been derived[18] from pseudocontact shifts of caffeine protons induced by paramagnetic metals (Mn(III), Fe(III), Co(II), Cu(II)) in the porphyrins. Possible structures of the complex have been filtered by computer scanning techniques, based on the known distance and angular dependence of pseudocontact shifts. An acceptable planar structure (shown in the figure) with an inter-ring separation of 0.4 nm has been proposed, in which C-5 of caffeine sits above the metal ion of the porphyrin. Chemical shifts have also been used in a more qualitative manner to demonstrate self-association of penicillins[19,20] and hexachlorophene[21] in solution.



Structure-affinity Correlations - A very thorough analysis of proton chemical shifts has been described[22,23] for complexes between vancomycin, an antibiotic isolated from Streptomyces orientalis that interferes with the biosynthesis of bacterial cell walls, and N-acetylated peptides. Association constants for these complexes can be determined from the composition dependence of proton chemical shifts of weakly interacting groups, such as the three methyls in acetyl-D-Ala-D-Ala. Chemical shifts characteristic of the bound peptide can also be determined by suitable extrapolation of the chemical shift data. Measurements of binding constants and bound site chemical shifts as a function of pH have shown that the stability of the complex results largely from electrostatic attraction between the peptide carboxylate anion and the vancomycin N-methylleucine cation. A detailed structure-affinity correlation has been established by studying association constants for a number of peptides and comparing these with the bound site chemical shifts at various positions along the peptide backbones. Only a single functional group (the Ala(1)-methyl) has a constant chemical shift in the bound site in each of the complexes studied. This methyl appears to be immobilized in a cleft of the antibiotic, while the remainder of the peptide chain assumes different configurations with respect to vancomycin for different peptides. The large variation of bound site shifts upon substitution shows that binding cannot be described as a series of additive

interactions of successive functional groups as is often assumed in structure-activity relationships.

Drug Binding to Phospholipids - Complexes between epinephrine and sonicated vesicles of several lipids have been studied[24] in order to assess possible lipophilic interactions between the hormone and phospholipids at the membrane receptor site. Chemical shifts in these complexes are vanishingly small. Therefore binding was monitored using spectral linewidths, W, which are directly proportional to the average rotational mobility of the drug or phospholipid molecule. Formation of a labile complex results in an increase in linewidth or, equivalently, a decrease in the transverse relaxation time, T_2, which may be defined by $T_2=(\pi W)^{-1}$. T_2's of epinephrine protons decreased much more rapidly in phosphatidylserine vesicles than in vesicles of phosphatidylcholine or phosphatidylethanolamine. This points strongly to ionic association since the former is anionic and the latter two are zwitterionic. Hydrophobic interactions are probably unimportant since T_2's of the epinephrine aromatic protons and protons of the lipid hydrocarbon tails are much less affected than the epinephrine NCH_3 protons. Penicillin-G and ampicillin also exhibit binding affinity for phospholipids in sonicated vesicles[25], and binding affinity again decreases in an order similar to that for epinephrine: phosphatidylserine>phosphatidylcholine> phosphatidylinositol>lysophosphotidylcholine. In an attempt to relate lipid solubility to activity of these antibiotics, it was found that in each case penicillin-G was immobilized by the lipid to a greater degree than ampicillin.

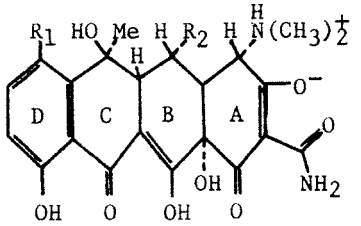
Relaxation time measurements provide the most generally useful n.m.r. probe of complex formation, and recent advances in Fourier transform techniques have greatly improved the reliability[26,27] and ease[28] of these measurements. Errors in T_1 measurements can be subtle and substantial[29,30] and have recently been systematically discussed. A rapid algorithm for computing T_1's that does not rely on the time-consuming "infinite delay" is available in program form[31].

The Intermolecular NOE Effect - The intermolecular nuclear Overhauser enhancement (NOE) has been used to demonstrate specific intermolecular associations in hormone storage complexes. This effect is observed as an intensity change of a resonance in one molecule when a closely neighboring nucleus in a different molecule is saturated. Recent theoretical work[32,33] has shown that positive enhancements result from magnetic dipole coupling in low molecular weight complexes, while negative enhancements may occur in macromolecular complexes, where rotational correlation times are comparable to 2π times the n.m.r. frequency. Negative enhancements also occur for exchangeable protons, for which magnetisation of the saturated site can transfer into the observed site by chemical exchange processes. Using NOE's and T_2's, binding sites have been mapped for oxytocin, vasopressin, and analog peptides in complexes with the pituitary binding protein neurophysin II[34,35]. The results indicate electrostatic binding between a protonated α-amino group of the peptide and a side-chain carboxyl of the protein. Close hydrophobic association of the single tyrosine of neurophysin II and an aromatic ring of the bound peptide was also demonstrated.

The solvent exposure of specidic protons of gramicidin-S[36] and angiotensin[37] has been studied by the same technique. P.m.r. difference spectra of angiotensin when the solvent (^1H_2O) resonance is saturated show positive enhancements for nonexchangeable exposed groups (His C_2H, His C_4H, Tyr o-CH Phe C_6H_5) and negative enhancements for those NH protons that exchange rapidly with the solvent.

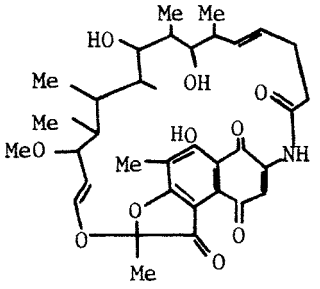
These homonuclear NOE experiments have been performed using a "rapid scan correlation" n.m.r. technique[38,39] that gives the sensitivity enhancement of Fourier transform n.m.r.[40], and yet is as convenient as traditional continuous wave n.m.r. for applying the homonuclear decoupling frequency and for suppressing the solvent peak. Several new methods for suppressing the solvent peak in pulsed-Fourier transform n.m.r. have been proposed[41-43].

Ionophore Antibiotics - Cation binding to the tetracycline antibiotics, aureomycin(I) and terramycin(II) has been studied by proton[44] and carbon[45,46] n.m.r. in DMSO solution. These antibiotics are believed to inhibit protein synthesis by binding, possibly through a metal ion, to bacterial ribosomes. Mg(II), Ca(II), La(III), and several paramagnetic ions bind strongly to a single site near the three carbonyls on the A ring, although the specific coordinating atom(s) could not be located. The macrocyclic antibiotics rifamycin-S (III)[47], valinomycin (IV)[48-52], nonactin(V)[53], and their analogues[52,53] also exhibit substantial C-13 chemical



I: R_1=Cl, R_2=H

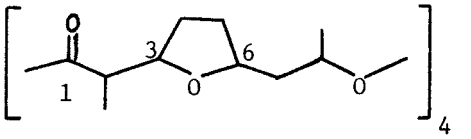
II: R_1=H, R_2=OH



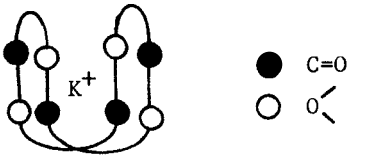
III:

IV: [(D-Val-L-Lac-L-Val-D-HyIv)]$_3$

HyIv=α-hydroxyisovaleric acid
Lac = lactic acid



V:



VI: [(L-Val-L-Pro-L-Pro-L-Ala-L-Phe)]$_2$

shifts at specific carbons in their metal complexes and these have been useful in structure assignments. Valinomycin shows high K^+ to Na^+ selectivity and binds K^+ with octahedral coordination through its six valine carbonyl oxygens[48]. The carbonyl carbons are shifted downfield by 1.5-4.5 ppm due to displacement of negative charge from carbon to oxygen in the complex[49]. An asymmetric coordination sphere occurs in antamanide(VI)[49], in which only the two valine carbonyls (δ_C=2.3 ppm) are directed toward Na^+, while four others (δ_C=0.5-1.5 ppm) have a C=O···Na^+ bond angle considerably less than 180° and approach the metal less closely.

Carbon-13 chemical shifts in metal complexes of nonactin indicate that this cyclic antibiotic forms similar complexes with many univalent cations (Na^+, K^+, NH_4^+, Rb^+, Cs^+) as well as with Ba(II)[53]. In this case however the complex is cubic, and cation coordination occurs to four carbonyl oxygens and four ether oxygens. Carbon-1 is shifted downfield by 2-4 ppm as expected from inductive effects, while carbons-3 and 6 are shifted by about 2 ppm.

The NH_4^+ complexes of nonactin and valinomycin have also been studied by proton n.m.r.[54] In the valinomycin complex spin coupling of ^1H-^{14}N causes differential relaxation of the inner and outer components of the NH_4^+ proton triplet. This effect permits an independent determination of the rotational correlation time and ^{14}N quadrupole coupling constant of NH_4^+ and shows that(1) NH_4^+ is bound rigidly with respect to internal rotation in the complex and (2) that distortion of the metal coordination sphere from the ideal geometry is much greater for valinomycin than nonactin.

Kinetics of Ion Transport - The kinetics of membrane transport of Pr(III) ions by the nigericin-type ionophores (A23187 and X537A) has been studied using sonicated dipalmitoyl lecithin vesicles[55]. In the absence of ionophore, Pr(III) is exposed only to $N(CH_3)_3$ groups on the outer surface of the membrane and selectively shifts these downfield. Addition of ionophore then produces a time-dependent shift of the inner resonances as Pr(III) is transported to the intravesicular aqueous phase. Transport occurs over tens of minutes and the shift can be used to quantify the kinetics.

Lanthanide Shift Reagents and Ion Resonances - Several other ion binding studies involving Lanthanide shift reagents have been reported, including conformational studies of the metal complexes of reserpine[56] and cyclic AMP[57]. In addition, quadrupolar resonances of various anions and cations have been used to characterize binding sites in proteins and amino acids [58-62]. For example, the ^{23}Na resonance has been used[63] to study K^+ inhibition of Na^+ binding to the transport site of Na^+,K^+-ATPase (the membrane pump enzyme). The ^{205}Tl resonance has been used to measure the distance (4.0+0.1 Å) between this site and the Mn(II) ion, which is bound to the enzyme at the site of ATP hydrolysis[64]. The observation of less common resonances has in the past required special probes or the use of wide-line n.m.r. spectrometers, but with the recent development of inexpensive broad-band probe inserts[65] and amplifiers[66], the observation of these nuclei is certain to become routine in many laboratories.

Serum Albumin Complexes - Binding of drugs by the plasma protein, serum albumin, plays an important role in their transport, distribution and elimination. This role has motivated several recent e.s.r.[67-69] and n.m.r.[70] studies of the molecular mechanism of binding. The acidic drugs flufenamic acid, phenylbutazone, sulfaethidole, oxyphenbutazone and warfarin produce conformational changes in spin-labeled BSA that loosen the secondary structure and possibly lead to exposure of additional binding sites[67]. Cationic drugs show very different conformational effects. Three basic tricyclic drugs lead to decreased mobility of the binding sites while three nontricyclic basic drugs produce no obvious conformational effects (possibly because the latter interact relatively weakly with BSA). Spin-labeled polyaromatics have been found[69] to bind rigidly to BSA in a region of intermediate polarity that has been interpreted as a "hydrophobic pocket". This picture shows marked similarity to a recent study, based on halogen n.m.r. resonances, of anion binding to HSA[55]. The "strong" anion binding sites are stabilized by dispersion forces and are most simply explained by charge neutralisation inside a hydrophobic cleft. The relation between these binding sites and those of fatty acids, which are largely hydrophobic but also contain a cationic moiety[72,73], is not clear.

^{19}F and ^{31}P Resonances - The ^{19}F and ^{31}P isotopes are both spin-1/2 and give intrinsically narrow, reasonably intense resonances that are convenient for monitoring ligand binding to proteins[74-79]. In this way the fluorine containing anesthetics fluothane and fluoromar were observed[80] to bind myoglobin and hemoglobin. The ^{19}F linewidths are dominated by dipolar coupling to the paramagnetic iron of the heme group and imply Fe(III)-^{19}F distances of 5-7 Å, which probably indicates ion coordination through oxygen and halogen atoms in the two anesthetics. A detailed theoretical study[32] of the dependence of ^{19}F relaxation times on rotational correlation times in protein complexes has appeared. The heteronuclear NOE (e.g., the enhancement of ^{19}F or ^{31}P when protons are saturated) falls to zero in a known manner with increasing correlation time and can be used to characterize molecular motion in the bound site. Chemical shifts of ^{19}F span a large range and are particularly sensitive to solvent effects. This fact has led to a suggestion[81] that the shifts provide a reliable probe of the ^{19}F environment in the complex. ^{19}F shifts of F_3SDS complexes with BSA and other proteins supports an apolar micellar environment of the surfactant at high binding numbers.

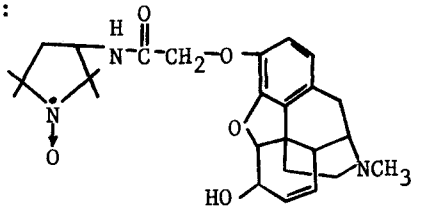
Drug Binding to Receptors - Potentially the most significant application of magnetic resonance to pharmacology is the direct observation of signals from receptor-bound drugs. The e.s.r. spectrum of spin-labeled morphine (VII) bound to isolated rat synaptosomes has been reported[82]. This compound does not cross the blood-brain barrier, but it retains some opiate activity when injected intracerebrally. Its specific binding to synaptosomes results in increased e.s.r. linewidths due to lengthened correlation times, and the binding can be blocked by nalorphine, which is known to displace morphine.

Spin-labels (VIII) and (IX) have also been developed as general probes of the outer surface of the cell membrane[83]. Although these spin-

labels are soluble in the membrane lipid phase, the net charge on the polar head groups prevents transport across the membranes. Thus the position of the radical in the hydrophilic portion of the labeled molecule ensures that only the environment of the outer surface of the membrane is sampled.

Effects on n.m.r. linewidths of epinephrine due to receptor binding in a crude suspension of mouse liver cells has been reported[84], based on the fact that line broadening is reduced when dichloroisoproterenol, a β-adrenergic blocking agent, is added. Unfortunately a similar experiment using rat liver plasma membrane was unsuccessful[85].

VII:



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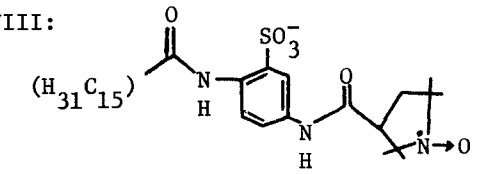

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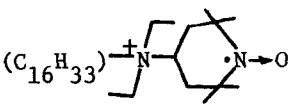

ATP Complexes in Intact Tissue - ^{31}P resonances show particular promise for observing ATP complexes in intact tissue. Proton and ^{31}P n.m.r. spectra of intact epinephrine storage vesicles (chromaffin granules) contain well-resolved resonances from epinephrine, ATP and the storage protein (chromogranin)[86]. A marked downfield shift in the ^{31}P resonance has been interpreted as evidence for an epinephrine-ATP storage complex, but this conclusion must be regarded as tentative in view of possible complex formation to chromogranin. ^{31}P resonances have also been used to demonstrate that Ca-ATP binds to the actin of rabbit muscle through its β and γ phosphate esters[87].

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