CONTRIBUTORS

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PREFACE

When a new editor takes over the reins there is a natural tendency to make visible changes in the new volume of a series. After consultation with many of our "customers," the new editor concluded that the volume in its present form is admirably serving its purpose. Consequently the reader will find only minor changes in format.

There is a growing tendency to cover certain very active areas yearly, while at the same time treating more mature fields on an every-two- or -three-year basis. This leaves room for the introduction of new topics on perhaps a one-time basis. In fact, just half of the present twenty-eight chapters deal with topics not included in the previous volume.

The sections, Topics in Chemistry and Topics in Biology contain chapters which are often more provocative and less drug oriented than the rest. In the latter section the emphasis on the rapidly maturing field of immunology is deliberate; some of these chapter topics will be developed in a planned way over the next two or three years.

The many dedicated hours devoted to the volume by authors and section editors are gratefully acknowledged by the editor and will become apparent to the reader as soon as he begins to read.

Suggestions for improvement and for new fields to cover are always welcome.

Kalamazoo, Michigan
June, 1972

Richard V. Heinzelman

AWARD ADDRESS

Inhibitors of Folate Biosynthesis and Utilization -- Evolutionary Changes As a Basis for Chemotherapy

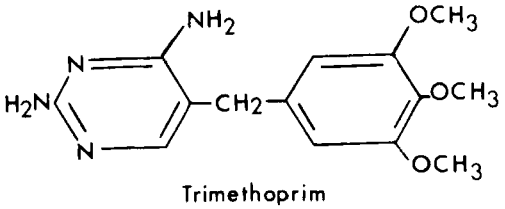
George H. Hitchings
Wellcome Research Laboratories, Research Triangle Park, N. C.

Fourth Award in Medicinal Chemistry, Thirteenth National Medicinal Chemistry Symposium of the American Chemical Society, Iowa City, Iowa, June 18-22, 1972

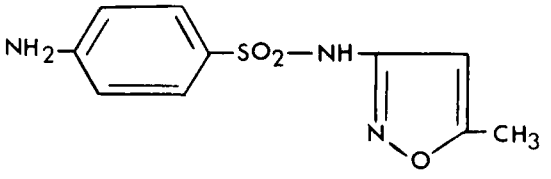
The exploration of enzymes and metabolic pathways by means of antimetabolites has been productive of both new medicinal agents and advances in fundamental knowledge[1].

One of the implications of such an approach to chemotherapy is that it is necessarily uncommitted with respect to specific targets. It feeds on the knowledge that it itself generates, and takes full advantage of the new discoveries arising from basic work elsewhere. Its goal is the unveiling of biochemical differences that can be exploited for the development of new medicinal agents. The program with which your Medalist has been associated has been involved with diverse categories: leukemia, protozoal and bacterial infections, gout and organ transplantation. All of these comprise a single package of applications from studies of nucleic acid metabolism.

This essay will attempt to describe the method of working primarily through one example, selective inhibitors of the biogenesis and utilization of folates and their application to anti-microbial chemotherapy[2]. Specifically, the combination of trimethoprim and sulfamethoxazole is now available in some 60 countries of the world (with the notable exception of the United States) and has been used in some 25 x 10^6 courses of therapy with highly satisfactory results[3,4].



Trimethoprim



Sulfamethoxazole

Its origins can be traced to early findings of the present program, to the detection of antimetabolites that possess selective effects[5]. Substances were found that exhibited a consistent pattern of antimetabolic

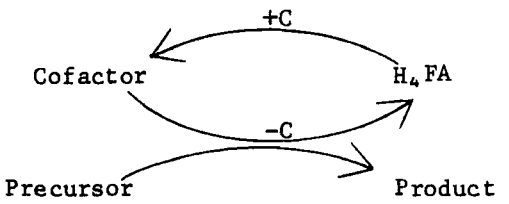
effects in microbial systems. Some were unusable as chemotherapeutic agents because of metabolic destruction or pharmacodynamic actions when they were tested in chemotherapeutic trials. Among them, however, was a sizable group that have "similar but distinct effects in different organisms" and it was felt that substances with such selective action could be regarded as leads to chemotherapeutic agents. Several possible modes of selective action were recognized:

> "(1) The compound may possess several active centers which allow it to block, more or less independently, different biochemical reactions in different tissues; (2) a single reaction may be blocked in all organisms but this reaction may be of relatively greater importance to one organism than another; (3) the dissociation of the inhibitor-cell receptor complex may vary widely from organism to organism; or (4) the reaction blocked may be one which occurs only sporadically in nature as an obligate biochemical mechanism"[5].

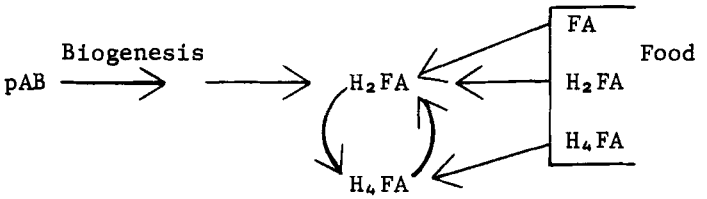
Trimethoprim is an outstanding example of the third mechanism stated above. It inhibits the dihydrofolate reductase of Escherichia coli at a concentration of ca. 10^{-9}M, while a concentration of nearly 10^{-4}M is required for a comparable inhibition of human liver dihydrofolate reductase[6].

The rationale for its use in combination with a sulfonamide is quite simply stated. The target is the microbial pool of tetrahydrofolate cofactors that are essential to the metabolism and multiplication of the microbial cell. Such cofactors occur ubiquitously and perform essential functions in all cells: in the biosynthesis of purines and thymine, the synthesis of serine and the methylation of homocysteine to form methione. Microorganisms employ similar reactions in the biosynthesis of riboflavin, pantothenate, thiamin and dihydrofolate itself. All of these latter metabolites have become "vitamins," i.e., the mechanisms for their biogenesis have been lost during the course of evolution, and higher organisms must have them preformed from food. Perhaps this in itself makes the microorganism the more sensitive to interference with the capabilities of the tetrahydrofolate pool. At some point, for example, reduction of the tetrahydrofolate pool results in inhibition of folate biosynthesis, a vicious cycle effect[7].

COFACTOR ACTIVITY

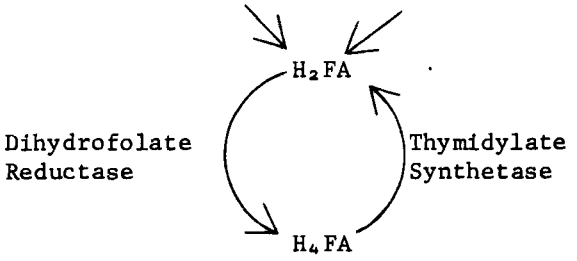


The selective effects of sulfonamides rest on a similar base. Sulfanilamide, and its relatives, act as antagonists of p-aminobenzoic acid, a building block of the folate molecule, and thus inhibit the biogenesis of dihydrofolate -- the primary product of the biosynthetic reaction[8]. Since man requires preformed folate, this antimetabolic effect is directed at a reaction that is present in the parasite and absent from the host. This, however, is only half the explanation of the chemotherapeutic activity of sulfonamides. Their action is contingent also on the inability of pathogenic microorganisms to incorporate preformed folates from the environment and so to by-pass the sulfonamide block. The ability to use preformed folates in evolution must have preceded the deletion of the biosynthetic route. Pathogens continue to synthesize folates de novo because they have failed to develop the ability to take advantage of the ample supplies of folates in body fluids[9].



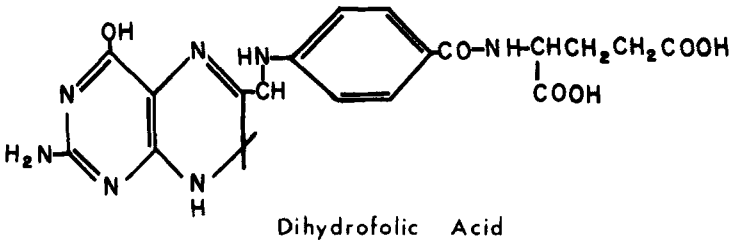
The effect produced by a sulfonamide on the microorganism's tetrahydrofolate pools is a depletion contingent on slow attrition and dilution through cellular division. Eventually, the pool falls to a level that will no longer permit multiplication, and the organism passes into a resting phase. The sulfonamide is thus bacteriostatic in its action.

Trimethoprim, by blocking the reduction of dihydrofolate, reduces the tetrahydrofolate pool more rapidly. In part, the attrition of the tetrahydrofolate pool is assisted by the activity of thymidylate synthetase, since in this reaction the cofactor not only transfers a one-carbon fragment (methylene) but contributes to the reduction of the methylene group to methyl, and is itself oxidized to dihydrofolate. The effect of trimethoprim is thus to trap folates in the dihydro- state, and the functional tetrahydrofolate pool is depleted.

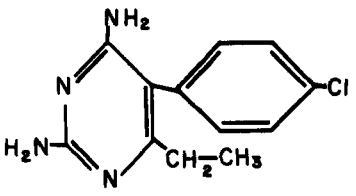


Trimethoprim, however, is a competitive inhibitor of dihydrofolate reductase[10]. The accumulation of dihydrofolate, through continuing biosynthesis and reoxidation of tetrahydrofolate, tends to increase the metabolite:antimetabolite ratio and diminishes the effectiveness of the blockade. Conjoint application of a sulfonamide, however, removes the source of new dihydrofolate and improves the effectiveness of the inhibition. In practice the simultaneous use of the 2 inhibitors results in a 5-10-fold potentiation, broadening of the spectrum of action, a decreased liability to the development of resistance, and a conversion of bacteriostatic to bactericidal effects[9].

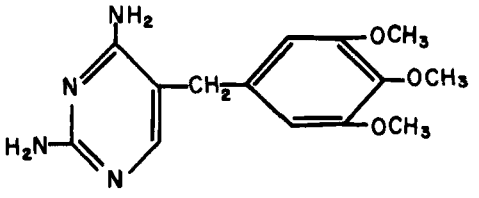
Trimethoprim emerged from a group of diaminopyrimidine derivatives which were viewed "as structural analogues of folinic acid of a remote sort" that were selective because they could have a "differential affinity...for the receptors of the parasite..."[11]. In the beginning they were characterized as substances that interfered with the utilization of folic acid by lactic acid bacteria. With the isolation and characterization of dihydrofolate reductase, their target was identified. The application of a spectrum of inhibitors to a selection of partially purified reductases[6] documented their mechanism of action, and revealed with clarity the magnitude of their differential affinities for the reductases of different species. Trimethoprim represents the culmination of an effort to tailor a molecule for maximum binding to bacterial reductases with minimum binding to mammalian reductases[9,12].



Dihydrofolic Acid



Pyrimethamine



Trimethoprim

Its selectivity has important implications for both chemotherapy and for evolution. The most probable interpretation of its action is that it is bound partly within and partly outside the active center of the enzyme. It thus differs in locus of binding from a structural analogue of folic acid, such as methotrexate, which has all the binding groups of the substrate and would thus be expected to bind in proportion to the substrate binding. The implication of selective binding in regions of the enzyme near to, but not in, the active center, is that evolutionary changes have been much more active in these supporting structures than in the active center itself. Dihydrofolate reductase thus falls in line with hemoglobins and other proteins, now known to exist in manifold variants that retain, in the main, the function of the primitive protein. It is being recognized that mutational changes are occurring at rates that would have been regarded as improbably enormous only 5 years ago, but that the bulk of such mutations are neutral[13]. An exciting chapter in the history of dihydrofolate reductase still lies ahead when studies on the sequence and conformation of the enzyme from different sources will permit comparative studies.

For chemotherapy, trimethoprim and its relatives carry the implication that selective inhibition of any function may be possible, and that the most probable access to selective effects may lie in the supportive structures of the cellular receptors.

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Section I - CNS Agents

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West Point, Pennsylvania 19486

Chapter 1. Antipsychotic and Anti-anxiety Agents

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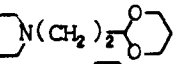
Introduction - Despite continuation of the extensive research efforts in this field, no fundamentally new antipsychotics and anti-anxiety agents emerged in 1971. However, the ever expanding investigations of the monoaminergic, particularly catecholaminergic, pathways in the brain and of the effects of drugs upon them suggest that this approach, which was so successful in setting the stage for the discovery of L-dopa for the treatment of parkinsonism, will eventually lead to a better understanding of the presently available drugs and to more effective or selective psychotropic agents. An important clinical development was announced - the resolution of the controversy over the use of Li_2CO_3 as prophylactic treatment of manic-depression.[1] Results of an extensive 4 year study to be reported by NIMH have confirmed the belief long held by some clinicians that daily use of the drug is effective in preventing broad swings in mood in patients with this disorder. A number of books and reviews treating the biochemical,[2-8] pharmacological,[3-6] clinical[3,4] and structure-activity[9,10] aspects of the antipsychotics and anti-anxiety agents have been published.

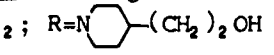
Tricyclic compounds with a six-membered central ring - Newer phenothiazine derivatives with clinical antipsychotic efficacy included oxaflumazine (1a),[11] a potent cataleptogenic in animals,[12] pipotiazine (1b, RP 19,336),[13] which has a pharmacological profile similar to that of fluphenazine, and a related piperidinol SA 124 (1c).[14] Extensive studies indicated clospirazine (1d, APY-606),[15,16] had clinical antipsychotic activity equivalent to that of chlorpromazine.[17]

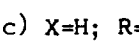
Several fatty acid esters of alcoholic phenothiazine derivatives, e.g., pipotiazine (1b) undecyclenate and palmitate, fluphenazine enanthate[18] and decanoate,[19] and perphenazine enanthate, as well as the related thioxanthene, flupenthixol decanoate, found clinical utility in antipsychotic maintenance therapy.[20] These esters can afford advantages in absorption to enable attainment of high drug-levels in the CNS[21] and to provide clinical efficacy of 2 to 4 weeks duration following a single injection.[20]

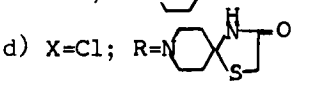
The most effective of a series of phenothiazines having diazabicyclo[4.4.0]decane and -[4.3.0] nonane systems in the side chain was 1e. In rats 1e was similar to perphenazine; it blocked conditioned avoidance response (CAR) at 0.5 mg/kg, sc.[22] Imiclopazine (1f), 5014 (1g) and 5023 (1h) were the most potent compounds in a series of imidazolone and oxazolidone derivatives of phenothiazine and 1-azaphenothiazine; they were more potent than perphenazine in tests for sedation and antiaggression in mice and as antiemetics in dogs.[23]

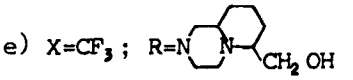
1

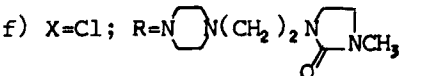
a) X=CF_3; R= 

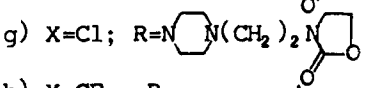
b) X=$SO_2N(CH_3)_2$; R= 

c) X=H; R= 

d) X=Cl; R= 

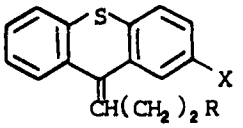
e) X=CF_3; R= 

f) X=Cl; R= 

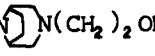
g) X=Cl; R= 

h) X=CF_3; R=same as in g

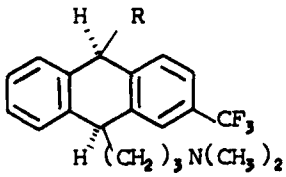
The most effective member (2a) in a series of amine-altered congeners of unsaturated thioxanthene antipsychotics was nearly equipotent with chlorpromazine in blocking CAR in rats and in depressing spontaneous motor activity (SMA) in mice.[24] Another thioxanthene (2b, prothixene) decreased SMA in mice only at high doses; however, it caused marked hypocholesteremia in rats.[25] A dihydroanthracene, SK&F 28175 (3a), produced pharmacological responses characteristic of both neuroleptic and antidepressant drugs whereas a demethyl analog, SK&F 25971 (3b), caused only chlorpromazine-like effects.[26]



2

a) X=CF_3; R= 

b) X=$OCH_2C_6H_5$; R=$N(CH_3)_2$



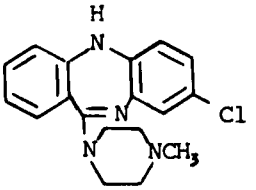
3

a) R=CH_3

b) R=H

Tricyclic compounds with a seven-membered central ring - A dibenzodiazepine, clozapine (4, HF-1854), was effective in psychotic patients;[27] however, it produced neither neuroleptic-like (e.g., catalepsy, antiemetic) actions in animals[28] nor parkinsonism-like symptoms in man. A derivative of dibenzthiepin, GP 45795 (5a), was more potent than chlorpromazine in various animal tests for neuroleptic activity and it antagonized amphetamine- and tetrabenazine-induced responses.[29] Clinically, 5a improved

chronic schizophrenics.[30] Among a series of related structures, norocto-clothepin (5b), oxometothepin (5c) and oxyprothepin (5d) had neuroleptic-like activity equal to, or greater than, that of octoclothepin in mice.[31] Clinical antipsychotic properties were reported[32] for 5d. Dehydroclo-thepin (6), one of a series of dibenzthiepins and dibenzoselenopines, was more potent than perphenazine in rotating rod and catalepsy tests in mice; however, toxicity and instability in aqueous acid are possible deterrents to its clinical investigation.[33] Two unsaturated congeners of peradi-thepin,[15] 7a and 7b, were substantially more potent than the parent in a rat catalepsy test.[34]



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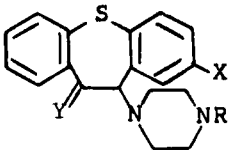
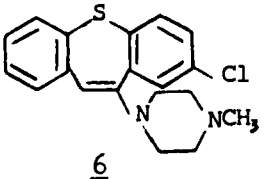


5

a) X=Cl; Y=O; R=CH_3
b) X=Cl; Y=H_2; R=H
c) X=OCH_3; Y=H_2; R=$(CH_2)_3OH$
d) X=SCH_3; Y=H_2; R=$(CH_2)_3OH$



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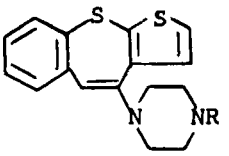
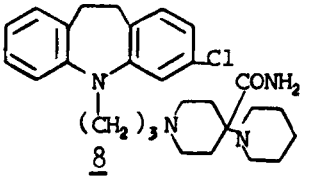


7

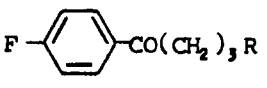
a) R=CH_3, b) R=$(CH_2)_3OH$



8

A dibenzazepine, "3-chlorocarpipramine" (8, Y-4153), was effective in assays of neuroleptic activity (0.2-1X chlorpromazine) and it is claimed to lack catoleptogenic properties.[35]

Butyrophenones and related compounds - The butyrophenone 9a was effective in the therapy of schizophrenics and caused only mild side effects;[36] it selectively blocked CAR (0.1X trifluperidol) in mice. AHR-2277 (9b) was more potent than chlorpromazine, but less potent than haloperidol, in suppressing CAR in mice and cats in doses below those producing obvious motor defects.[37] A related compound AHR-1900 (9c) elicited neuroleptic-like behavioral effects in mice, rats and squirrel monkeys.[38] In a series of butyrophenones bearing unsaturated amino substituents, most potent CNS depressant activity in mice was noted with 9d.[39] Abbott-30360 (9e) combined neuroleptic properties with analgetic potency approximately equal to that of morphine.[40] The most potent compound (10) in a series of methyl-branched aminobutyrophenones was only weakly depressant in mice.[41]

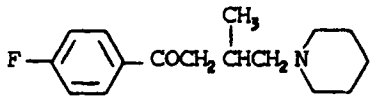


9

9a) R=-N⟨⟩-OCONHCH(CH_3)$_2$
9b) R=-N⟨⟩-CO-⟨⟩-F
9c) R=-N⟨⟩-OC_6H_4-2-OCH_3

9d) R=-N⟨⟩

9e) R=-N⟨⟩-F
NH



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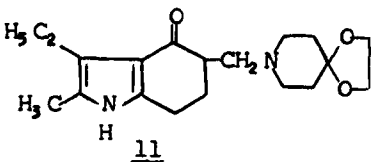

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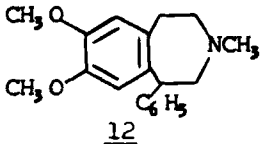
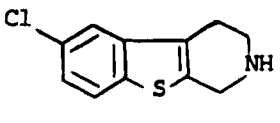


12

Other structures with antipsychotic activity - A molindone analog, AL 1612 (11), demonstrated antiemetic and neuroleptic properties in animals;[42] however, clinical antipsychotic activity was accompanied by a high incidence of extrapyramidal side effects.[43] Combined clinical antipsychotic and antidepressive actions were noted for sulpiride.[15] Sch-12,679 (12) reduced aggressive behavior in mice and monkeys; however, a clinical study indicated it is not a classical neuroleptic.[44] Chronic schizophrenics were improved by D-penicillamine.[45] Prostaglandin E_2, like chlorpromazine, decreased CAR in both naive and trained rats.[46] A benzothienopyridine, EX 11-349 (13), decreased SMA in mice and was effective in a fighting mouse test (ED_{50} 8.9 mg/kg, po).[47] Several members of a series of 1-acylated-1,2-dihydroquinolines 14 antagonized amphetamine actions, blocked a CAR, and caused neuroleptic-like effects in mice and rats.[48] The cis-, but not the trans, aminoalcohol (15) was effective in several pharmacological tests for neuroleptic activity.[49] In mice, the benzothiazinone 16 was the most potent of a series of compounds in causing depression, catatonia and decreased SMA.[50]



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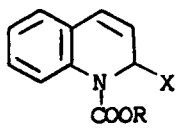
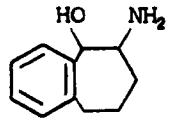


R=alkyl, $CH_2C_6H_5$

X=H, OCH_3, OC_2H_5, SCH_3, $SCH_2CH{=}CH_2$

14



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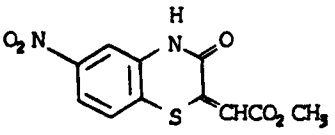
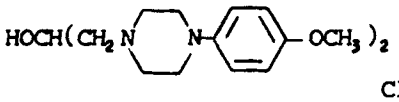
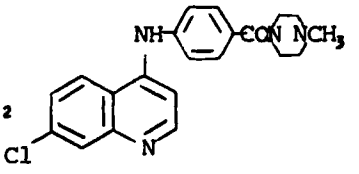


16



17

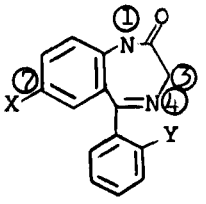


18

Benzoquinolizine-like (short-acting, selective) depletion of central catecholamines was produced by Ro-8-2580 (17)[51] and U-20,057 (18),[52] compounds causing neuroleptic-like effects in animals.

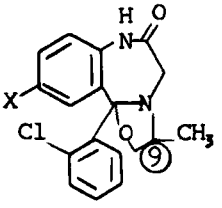
Benzodiazepines and related compounds - The chemistry and some aspects of the relationship between the structure of 1,4-benzodiazepines and their anti-anxiety activity were reviewed in 1971.[53] In clinical studies, demethdiazepam (19a, Ro-5-2180, A-101) was more effective than diazepam for the treatment of anxiety;[54] however, Ro-5-3350 (19b) was less

effective.[55] Several pharmacological[56,57] and clinical[58] studies indicated lorazepam (19c, Wy-4036) is a potent anti-anxiety agent with a sedative-hypnotic component. Sch-12,041 (19d) showed evidence of anti-anxiety activity in an uncontrolled study.[59] Methyloxazepam (19e) was the most potent of a series of benzodiazepines in potentiating methamphetamine-induced stereotyped behavior in rats.[60] In monkeys, Ro-5-4200 (19f) was effective in causing EEG and behavioral effects (20X diazepam).[61] Various 7-sulfonamido-1,4-benzodiazepines (19, X=H_2NSO_2, $(CH_3)_2NSO_2$, CH_3SO_2NH; 1-H or CH_3) had antinicotine activity in mice, but they were much less potent than diazepam.[62] Ro-5-6901/3 (19g) was the most effective of 8 benzodiazepines tested for anticonvulsant activity in mice.[63]



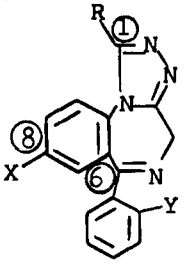
19

a) X=Cl; Y=H
b) X=Br;  =2-pyridyl
c) X=Cl; Y=Cl; 3-OH
d) X=Cl; Y=H; 1-CH_2CF_3
e) X=Cl; Y=H; 1-CH_3; 3-OH
f) X=NO_2; Y=F; 1-CH_3
g) X=Cl; Y=F; 1-$(CH_2)_2N(C_2H_5)_2$



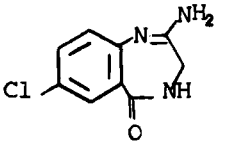
X=Cl, Br, NO_2

20



21

a) R=CH_3; X=Cl; Y=F
b) R=CH_3; X=Y=Cl
c) R=CH_3; X=Cl; Y=H
d) R=Y=H; X=Cl
e) X=Y=H; R=CH_3
f) X=H; Y=Cl; R=CH_3



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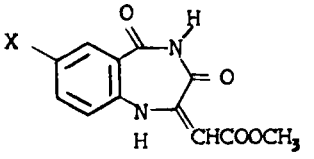
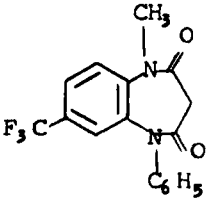


X=Halogen, CH_3, CH_3O

23



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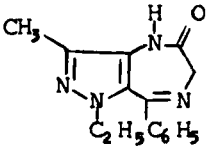


25

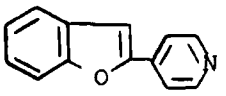
In an extensive series of benzo[6,7]-1,4-diazepino-[5,4-b]oxazoles and thiazoles related to oxazolazepam,[15] several 9-methyl derivatives (20) were more potent than diazepam as antagonists of bemigride-induced convulsions in mice.[64]

Triazolobenzodiazepines (21) demonstrated potent and selective activity in various pharmacological tests generally thought to indicate

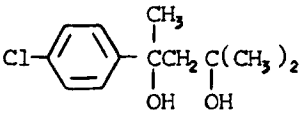
anti-anxiety activity. Structure-activity data[65] for a series of these compounds indicate substitution of the 8-position with electron-withdrawing groups generally enhances potency, as does 1-methyl substitution. Substitution of the 1-position with alkyl groups larger than ethyl as well as introduction of a para-methoxyl into the 6-phenyl moiety caused a marked decrease of potency.[66] Substitution of Cl or F at the ortho position of the C-6 phenyl ring gave compounds with dramatically enhanced activity in many tests,[65] e.g., 21a and 21b were very potent (30X diazepam) upon s.c. administration to mice in an anti-pentylenetetrazol test. Extensive pharmacological[66,67] and biochemical[68] data are reported for D-65-MT (21c), D-40-TA (21d), U-31,957 (21e) and U-35,005 (21f).

A benzodiazepinone CT 5104 (22) was considerably less protective than chlordiazepoxide versus electroshock-induced convulsions in mice.[69] The benzodiazepindiones 23 were ineffective as antagonists of pentylenetetrazol-induced convulsions in rats.[70] In humans, ORF 8063 (24) elicited electrophysiological and behavioral changes similar to those produced by diazepam.[71] The pyrazolodiazepine CI 683 (25) had clinical anti-anxiety activity. In rats, it counteracted conflict-induced depression and antagonized pentylenetetrazol-induced convulsions.[72]

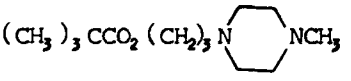
Several miscellaneous structures were examined for anti-anxiety activity. Propranolol was effective in LSD-induced anxiety in humans.[73] As both (+) and (-)-isomers produced tranquilization in rats, the action is not attributed to β-adrenergic blockade.[74] Although pyridarone (26) was the most potent member of a series studied for neurodepressant activity in animals, anti-anxiety action could not be confirmed in a clinical study.[75] Fenpentadiol (27) displayed anti-anxiety and stimulant properties in animals.[76] Meprobamate-like activity was noted in pharmacological studies of Inserm-54 (28).[77] The pharmacology of propanediol carbamates was reviewed recently.[78]



26

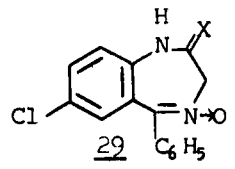


27

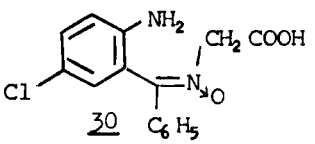


28

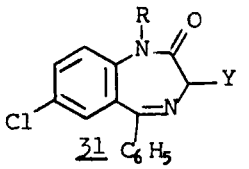
Metabolism of benzodiazepine derivatives - Biological half-life studies of chlordiazepoxide in man showed desmethylchlordiazepoxide (29a) and demoxepam (29b) to be major metabolites.[79] In dogs, metabolism of 29b involves two oxidative pathways. One leads to two phenolic products: 5-(4-hydroxyphenyl)- and 9-hydroxy derivatives. The other involves loss of the N-oxide function to give N-desoxy-29b, oxazepam, and N-desoxy derivatives of the phenolic products. Also, hydrolysis of demoxepam affords an "opened lactam" (30).[80] In man, metabolites of demoxepam were oxazepam conjugates, 30, and phenolic products.[81] The pharmacological effects of diazepam in mice were related to brain levels of its metabolites, particularly oxazepam.[82]



29 a) X=NH b) X=O



30



31 a) R=CH_2-⬡; Y=H b) R=Y=H c) R=CH_2-⬡; Y=OH

Metabolism of prazepam (31a) by human and animal liver preparations gave mainly desalkylprazepam (31b) plus a small amount of oxazepam, but no 3-hydroxyprazepam (31c).[83] In contrast, the principal urinary metabolites of prazepam in man were glucuronides of 31c and oxazepam and small amounts of 31b.[84]

Diazepam and phenolic diazepam derivatives were significant metabolites of medazepam in dog, rat and man.[85]

Hypotheses, Biochemistry and Pharmacology - The results of extensive histochemical mapping by Swedish investigators[3,5,7,8] of catecholaminergic (CA) pathways in the rat brain suggest that these systems may play crucial roles in the complex neural traffic which courses back and forth between brain stem centers, hypothalamus, thalamus, basal ganglia (e.g., neostriatum), limbic system and cortex, and provides the neural basis for perception, arousal, motivation and emotional behavior. The most clearly defined tract is a nigrostriatal dopaminergic (DA) pathway ascending from the substantia nigra to the neostriatum. Another DA system projects from the midbrain to the limbic system. Two noradrenergic (NA) pathways, originating in the reticular formation and ascending through the medial forebrain bundle, together innervate the hypothalmus, thalamus, areas of the limbic system and the cortex. Growing evidence indicates that the antipsychotics exert many of their pharmacological and clinical effects by actions on these CA neural systems. The EPS produced by these drugs clearly indicate an action on the basal ganglia. Lesions in the nigrostriatal pathway, attended by a loss of DA in the neostriatum, appear to be in large part responsible for the EPS of parkinsonism, and the EPS produced by reserpine probably are the consequence of the DA-depleting action of the drug.[5] Although other types of neuroleptics such as the phenothiazines and butyrophenones do not have an appreciable effect on brain levels of DA they too affect DA metabolism in the neostriatum. Various biochemical effects of these agents, e.g., an increased level of homovanillic acid, indicate that they accelerate the turnover of DA in the neostriatum. It is postulated that the antipsychotics block postsynatic DA receptors and that the resulting functional deficiency of DA activates a feed-back mechanism to stimulate synthesis of the transmitter.[2-5] The observation that the neuroleptics do not appreciably increase turnover of DA unless nerve impulse flow is intact seems to favor a postsynaptic mechanism of DA blockade[86] rather than a presynaptic mechanism[87] like that of reserpine. These drugs also increase turnover of DA in the neurons which project to the limbic system and some accelerate turnover of norepinephrine (NE) in the NA systems.[5,88] However, individual drugs

seem to differ considerably in their effects on the two types of neurons. In an extensive study of a variety of neuroleptics, it was found that perphenazine accelerated turnover of both amines, spiroperidol increased turnover of only DA and pimozide increased turnover of DA at low doses and NE at high doses.[88]

Rats with unilateral lesions of the nigrostriatal pathway have been used as a model for measuring the DA activity of drugs such as amphetamine and the DA blocking activity of antipsychotics in the neostriatum.[5,88-90] These animals display motor asymmetries and a tendency to turn toward the side of the lesion (ipsilateral turning). Amphetamine enhances the turning behavior and the neuroleptics antagonize this effect. If the neostriatum is lesioned or removed, the neuroleptics cause contralateral asymmetries. A similar mouse model has been developed and used to assess the activity of various antipsychotics.[91]

The characteristic cataleptogenic property of the antipsychotics has been thought to be due to an effect of these drugs in the neostriatum.[92-95] Consistent with this idea is the finding of a correlation between the potencies and time courses of action of drugs in producing catalepsy and in producing increased homovanillic acid levels in the neostriatum of rats.[93] However, a comparison of the effects of lesions in the substantia nigra, neostriatum or globus pallidus upon antipsychotic- and cholinergic-induced catalepsy suggests that integrity of the globus pallidus is important for the action of neuroleptics but not for the action of cholinergics.[94,95]

Antagonism of amphetamine- or apomorphine-induced stereotyped behavior is another characteristic effect of the antipsychotics. Most data favor the hypothesis that drug-induced stereotyped behavior is the result of DA activity in the neostriatum.[5,92] However, a comparison of the effects of lesions in the neostriatum or in the tuberculum olfactorium upon DA- and apomorphine-induced stereotyped behavior suggests that DA activity in the limbic forebrain may be important for this drug effect in the rat.[96]

The antipsychotics are also noted for their ability to attenuate brain self-stimulation. Animals with electrodes implanted in the septum (in the limbic system) or the medial forebrain bundle, through which CA neurons ascend to the limbic system, repeatedly press a lever to obtain electrical stimulation of these areas. Thus the "reward or pleasure" centers involved in this behavior may be located in areas of the limbic forebrain.[97] A study extending earlier work of Stein[97] shows that self-stimulation is accompanied by release of NE from NA terminals in the hypothalamus, preoptic area, and parts of the limbic system.[98] Another paper reports that self-stimulation also occurs when electrodes are placed in regions near the substantia nigra where DA neurons are located.[99] Thus DA as well as NA pathways may be involved in the effects of reward on operant behavior and in the attenuating effects of the neuroleptics on self-stimulation. This behavior is also attenuated when 6-hydroxydopamine, which causes degeneration of CA neurons and depletion of the amines, is

given intracisternally to rats.[100] Stein and Wise[101] have proposed a novel etiology of schizophrenia which involves the endogenous buildup of 6-hydroxydopamine and a resultant deterioration of NA pathways that mediate reward. They postulate that the antipsychotics produce their clinical effects by preventing the uptake of 6-hydroxydopamine into the NA neurons. Several criticisms[102-104] of this hypothesis and a rebuttal[105] have been published. These point out the problems of defining and characterizing mental disorders as well as some of the experimental and interpretational difficulties of research in this area.

Hypotheses regarding action of the antipsychotics at the cellular and molecular levels range from a view that the drugs interact non-specifically with neuronal and subcellular membranes to a postulate that they specifically block postsynaptic receptors of neurotransmitters, e.g., DA and NE. These drugs are potent surface-active agents and stabilize cellular and subcellular membranes and it has been proposed that they act like local anesthetics by altering membrane permeability and interfering with neural transmission.[106] Consistent with this view are observations that procaine, when injected into the neostriatum of cats, produced effects similar to those of haloperidol[107] and that ouabain, another agent which alters membrane function, produced many of the effects of the antipsychotics when given intraventricularly.[108] An *in vitro* study of the effects of promazine and thioridazine on membrane preparations suggests that inhibition of Na^+-K^+-ATPase by these agents is secondary to more general alterations of the cell membranes.[109] Another hypothesis proposes that the antipsychotics prevent the access of neurotransmitters to their receptors by forming a monomolecular film on the postsynaptic membrane,[110] but Horn and Snyder,[111] noting that the solid-state structure of DA is superimposable upon a portion of the solid-state structure of chloropromazine, suggest that the antipsychotics might selectively interact with CA receptors. The effects of the antipsychotics upon brain tissue levels of cyclic AMP, which could be the mediator of central CA actions, have been studied *in vivo* and *in vitro*.[112,113] Trifluoperazine[113] and haloperidol[112] were more potent than chlorpromazine in preventing the rise in cyclic AMP levels produced by norepinephrine or decapitation. The sulfoxides of the two phenothiazines,[112,113] promethazine,[113] and protriptyline[112] were much less potent than chlorpromazine.

If both antipsychotic and local anesthetic activity are the result of nonspecific interaction of drugs with membranes, the characteristic CNS actions of the antipsychotics must be due to a particular drug distribution into various brain regions, a selective affinity for certain neurons, or a differential susceptibility to drug action by various interneuronal organizations. It has been observed that different regions of the brain take up different amounts of chlorpromazine, but no difference in the affinity of the neuroleptics for tissue from various brain regions was detected in *in vitro* experiments.[106] A study of phenothiazines showed that the different potencies of the 1-, 2-, 3- and 4-chloropromazines in producing catalepsy is not due to differences in the availability of the four isomers in the brain.[114] In contrast, a careful study of four butyrophenones indicated that their molar concentrations in the brain were

about the same after administration of ED_{50} doses for antagonism of amphetamine- and apomorphine-induced stereotyped behavior.[115] Thus, if total brain levels of antipsychotics are an index of drug availability at sites of action, these limited observations suggest that the differences in the potencies of the antipsychotics can be accounted for by differences in their abilities to reach their sites of action in the case of the four butyrophenones but not in the case of the isomeric phenothiazines. Much more extensive studies correlating biological activity with total or regional brain levels of a variety of antipsychotics of diverse structure and potencies must be carried out before the significance of the structural requirements for antipsychotic activity can be understood.

Although there are some indications that Li_2CO_3 in some way affects CA metabolism,[116] little progress has been made in explaining its specific therapeutic effects in manic-depressive psychosis. Manic or hypomanic states sometimes develop in the course of L-dopa therapy, particularly in patients with a prior history of mania.[117,118] From the meager conflicting data available[118] it cannot be said yet whether or not Li_2CO_3 is effective in ameliorating L-dopa-induced mania.

Additional data support the idea that effects of anti-anxiety agents upon CA pathways may at least in part account for their clinical action. A study of the effects of chlordiazepoxide, diazepam and nitrazepam on CA neurons of non-stressed and stressed rats using histochemical and biochemical analyses of amines suggests that these drugs decrease impulse activity in NA neurons innervating the cortex and hippocampus and in DA neurons ascending to the neostriatum and limbic forebrain.[119] These results are consistent with electrophysiological observations that the anti-anxiety agents control abnormal activity in various components of the limbic system at doses which have little or no effect on the cortex.[120]

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Chapter 2. Antidepressives and Stimulants

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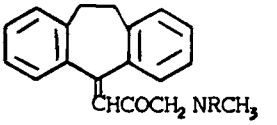
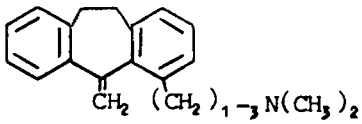
Introduction - As in past years, this chapter reviews stimulants together with antidepressives. This arrangement of topics seems not to be justified on clinical grounds for the spectra of clinical utility of the two categories of drugs are quite different. Is there any reason for treating the two subjects together other than one of convenience? Perhaps the answer is yes if the definition of "stimulants" is limited to amphetamine and pharmacologically related agents. Most research reported in the last year supports the long held belief that the antidepressives and the amphetamine-like drugs exert many of their pharmacological and clinical effects by actions on central monoaminergic, especially catecholaminergic, pathways. But the biochemical actions of the tricyclic antidepressives are still poorly understood and their relevancy to the therapeutic effects has not been established. From the extensive studies on amphetamine a more coherent, if not clearer, picture of its actions is emerging. A sign of progress is that one can now with some assurance describe amphetamine as a catecholaminergic agent with a pronounced dopaminergic component of action rather than as a centrally-acting sympathomimetic amine.

The 1971 literature describes the synthesis and pharmacological evaluation of numerous potential antidepressives, but presents no evidence that new agents superior to the established drugs are in the offing.

Tricyclic compounds with antidepressive activity - An amitriptyline derivative, SD 2202-01 (1a), exhibited a pharmacological profile similar to that of the parent.[1] Antidepressive and anti-anxiety properties were also noted for a monomethyl analog (1b).[2] Other amitriptyline relatives, some 5-methylene-4-substituted dibenzocycloheptenes (2), had little activity in a mouse antireserpine test.[3] A dibenzthiepin, dothiepin (prothiadine), was superior to amitriptyline in a double blind comparison for the treatment of depression.[4] Amoxapine (3, CL-67772), a dibenzoxazepine, although devoid of anticholinergic properties, produced imipramine-like activity in animals. Clinically, 3 was an effective antidepressive agent; however, side effects (insomnia, difficult urination) were encountered.[5] Several related dibenzodiazepines had antidepressive properties. SM-307 (4), an ethylsulfonyl analog of dibenzepin,[6] produced



1a: R=CH3
1b: R=H



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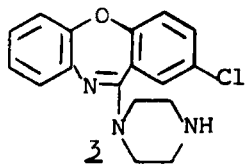

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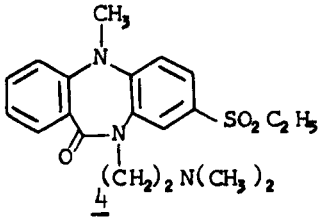
imipramine-like effects in animals.[7] A related pyridobenzodiazepine, UP-106 (5), also was active in various animal tests in which most antidepressives are effective and it demonstrated clinical activity.[8] The structurally similar U-17,660 (6) was the most potent antiallergic and antianaphylactic compound in a series of 5- and 10-aminoalkylated dibenzodiazepines.[9]



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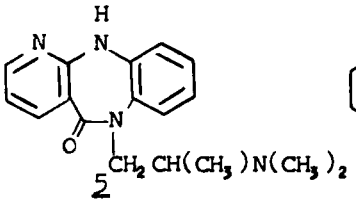

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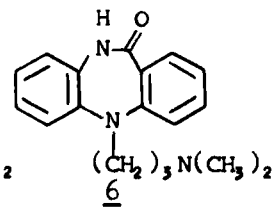

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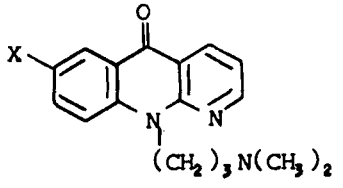
Antidepressive activity was noted for some tricyclic compounds having a six-membered central ring. A series of naphthyridones, C-29 (7a), C-42 (7b), and C-45 (7c), caused imipramine-like activity in animals.[10] The influence of 7c on central biogenic amine levels,[11] as well as its absorption, distribution and excretion,[12] was investigated in mice and rats. Several phenothiazine derivatives had antidepressive activity. In extensive clinical trials fluoracizine (8a), a CF_3 analog of chloracizine,[13] showed antidepressive efficacy equivalent to imipramine. It also produced anticholinergic effects.[14,15] Structure versus antidepressive activity relationships were reported for a series of phenothiazines and related compounds bearing a carbocyclic basic side chain. One of these compounds (8b) was considerably more potent than amitriptyline in preventing reserpine-induced ptosis in mice and rats.[16] Other phenothiazines with antidepressive actions included 8c, the 1-chloro-analog of chlorpromazine, which produced imipramine-like actions in mice and rats,[13] and the azaphenothiazine-10-thiolcarboxylate 9 which induced antidepressive-like symptoms (mydriasis, increased sensitivity to touch) in mice.[17] The antidepressive effects of phenothiazine derivatives with 1- or α-substituents may be associated with the steric influence of these groups in interfering with attainment of a nearly flat conformation by the tricyclic system.[13]



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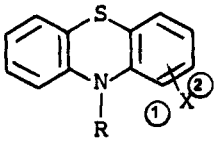
a) X=H
b) X=OCH_3
c) X=Cl



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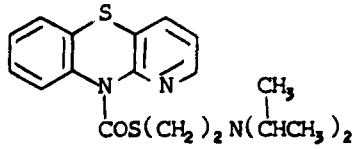
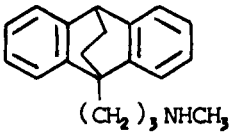
a) X=2-CF_3; R=$CO(CH_2)_2N(C_2H_5)_2$
b) X=2-Cl; R=trans-$CH_2N(CH_3)_2$
c) X=1-Cl; R=$(CH_2)_3N(CH_3)_2$



9

Other tricyclic compounds with antidepressive activity were maprotiline (10, Ciba 34276-Ba), which produced imipramine-like effects

in dogs[18] and blocked rat synaptosomal uptake of norepinephrine[19] (NE), and the pyridobenzocyclooctane (11), which antagonized tetrabenazine-induced ptosis in mice at ED_{50} 20 mg/kg, p.o.[20] In mice, the phenanthrene 12 had no antireserpine activity[21] and a 6,9-dihydro derivative of imipramine failed to antagonize tetrabenazine-induced depression.[22]



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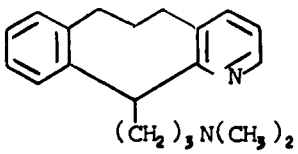

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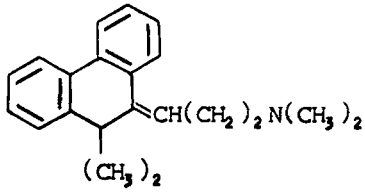
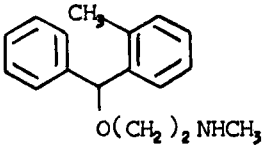


12

Several diphenylmethane derivatives had antidepressive activity. Tofenacin (13) was well-tolerated and effective in patients with various kinds of depression.[23,24] Like imipramine, some aminopropenes 14 reversed reserpine-induced hypothermia in mice.[25] The (-)-isomer of 15 caused clinical antidepressive actions, but these were accompanied by unwanted side effects.[26]



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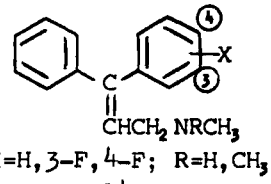

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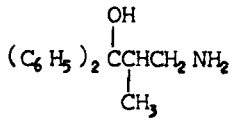
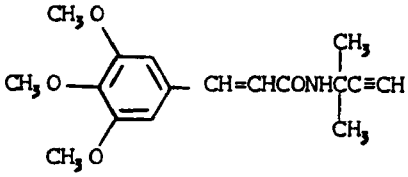


15

Other compounds with antidepressive activity - It has been suggested that rubidium salts (see below) may have antidepressive activity and a remarkable therapeutic response was noted in an open clinical trial.[27,28] Clinical antidepressive activity was produced by A-25794 (16).[29] A molindone relative, EN-3022 (17), was effective in several tests (antagonism of tetrabenazine-induced ptosis in mice; potentiation of pressor response to NE in dogs)[30] in which most antidepressives are active. Nomifensine(18) was more potent than imipramine in antagonizing reserpine-induced catalepsy in rats.[31] Quipazine (19) had a pharmacological profile similar to that of tricyclic antidepressives.[32] In a series of phenacylthioimidazolines, 20 was the most potent antagonist of reserpine-induced hypothermia in mice (12X imipramine).[33]



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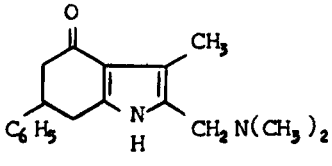

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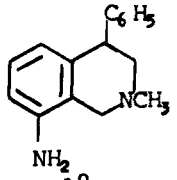

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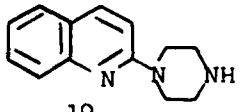

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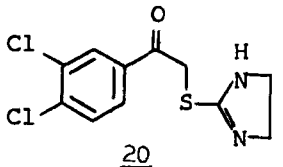
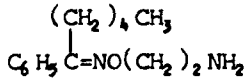


20

$(C_6H_5O)_2CHCH_2N(CH_3)_2$

21

Imipramine-like pharmacological activity, without anticholinergic properties, was noted for LG 152 (21);[34] it also prevented synaptosomal reuptake of NE.[35] Potent antitetrabenazine-induced ptosis activity, without anticholinergic effects, was produced by some ethers of hexanophenone oxime, e.g. 22.[36] The piperidinecarboxamide (23) was equipotent with amitriptyline in a test for dopa-potentiation in mice.[37] A series of phthalan derivatives (24) having a pharmacological spectrum similar to the tricyclic antidepressives was studied for inhibition of mitochondrial activity in intact yeast cells - a test which correlates with clinical antidepressive potency. Most potent activity was noted for Lu 3-047 (24a), Lu 3-009 (24b), Lu 3-074 (24c, 3X impramine)[38] and Lu 3-049 (24d, 4.3X imipramine).[39] Clinical antidepressive actions were noted for HT 3261 (25)[40] and the choline derivative panclar (26), which was an effective psychostimulant in patients with schizophrenia, depression and neuroses.[41] Other compounds with clinical antidepressive activity included WIN 27,147-2 (27), which was devoid of side effects at effective dose levels,[42] and CP 14.368 (28),[43] which was of doubtful value in humans and caused untoward side effects although it produced antidepressive-like actions in animals. Short-lived antidepressive activity was observed with L-dopa.[44]



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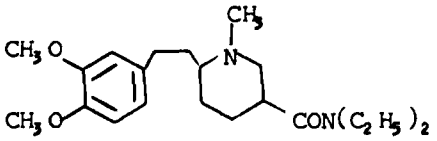

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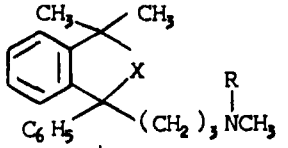
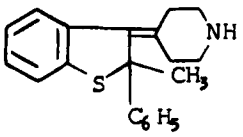


24

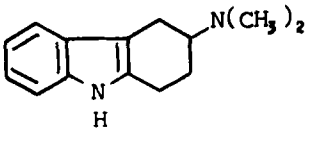
a) X=CH_2 ; R=CH_3
b) X=O; R=CH_3
c) X=S; R=CH_3
d) X=CH_2 ; R=H



25

$HOPO_3(CH_2)_2N(CH_3)_2$

26



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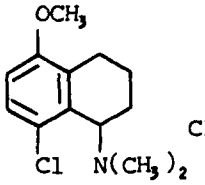

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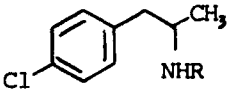
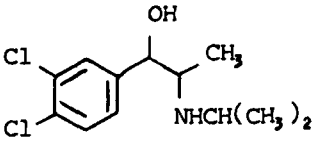


29a: R=H
29b: R=CH_3

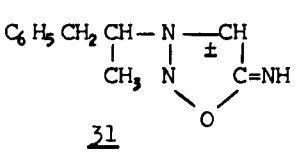


30

Several amphetamine derivatives displayed antidepressive properties. Fenfluramine, an anorectic drug which depresses motor activity, showed an imipramine-like profile of activity in various pharmacological tests in mice.[45] p-Chloroamphetamine (29a)[46] and its N-methyl derivative,

Ro-4-6861 (29b),[47] produced clinical activity resembling that of the tricyclic antidepressives. BW 65-54 (30) elicited antidepressive effects in animals; it potentiated responses to amphetamine, apparently by interfering with metabolic inactivation.[48]

A number of new MAO inhibitors were described in 1971; however, because of the limited clinical utility of these agents in the treatment of depression, they will not be reviewed in detail. Nonetheless, several of these substances are noteworthy. Sydnophene (31)[49] was included in a series of sydnonimines examined for MAO-inhibitory activity. It demonstrated mild stimulant and antidepressive actions in extensive clinical studies in Russia.[50] Another MAO-inhibitor, clorgiline (32, M&B 9302) had antidepressive activity; its clinical effects were similar to those of imipramine.[51] Proportions of two forms of MAO (Type A, B) were found to vary in different areas of rat brain. The type A enzyme, predominant in sympathetic nerves, acted on both 5-HT and tyramine and was inhibited by 32, whereas type B-MAO was insensitive to 32 and acted on tyramine, but not 5-HT.[52] In humans, 32, tranylcypromine and isocarboxazid increased 5HT, NE and dopamine levels in various brain areas; however, the effect of tranylcypromine was significantly greater than that of the other two on dopamine in the caudate nucleus and hypothalamus.[53] In vitro, Lilly 51641 (33), like harmaline, preferentially blocked MAO oxidation of 5-HT, whereas tranylcypromine and pargyline selectively inhibited the oxidation of tyramine.[54]



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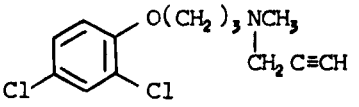

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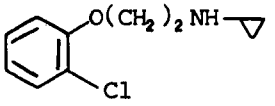


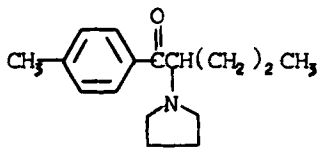
33

Central Stimulants - Structure-activity relationships of central stimulants were reviewed in 1971.[55,56]

The use of central stimulants, notably dextroamphetamine and methylphenidate, in the therapy of hyperkinetic disorders in children has increased. According to NIMH estimates more than 200,000, or about 10% of hyperactive children, received amphetamine treatment in 1969.[57] These agents reduce distractibility and promote the child's ability to focus on meaningful stimuli and to organize his bodily movements more purposefully.[58,59] Similar effects were elicited by treatment of untrainable hyperkinetic and aggressive dogs with dextroamphetamine.[60]

Newer amphetamine relatives included pyrovalerone (34), which was beneficial in therapy of chronically fatigued patients,[61] U-30,405A (35a) and U-28,926A (35b), which caused CNS stimulation in rhesus monkeys.[62] Amantadine produces amphetamine-like action (see below). An aminotetralin, ADT (36) afforded amphetamine-like effects but decreased motor activity in mice.[63] Adenosine had a psychoanaleptic effect in a mouse maze test.[64] Exploratory activity of mice was enhanced by P1961 (37).[65] Other compounds for which CNS-stimulating activity was observed in mice

included 4864 (38),[66] a benzocyclobutene (39),[67] several thiolactams,[68] and GYI 20238 (40).[69]



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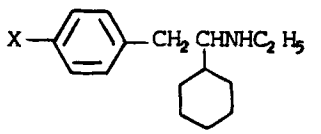
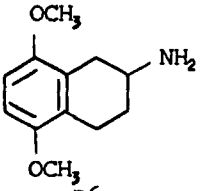


35a: X=Cl 35b: X=H



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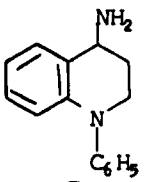

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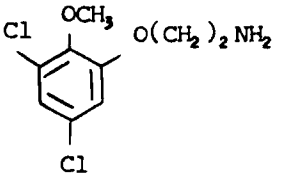

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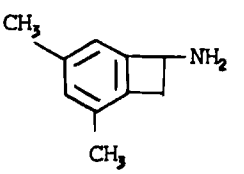

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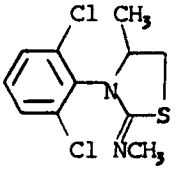

40

Several studies related to physical properties and metabolism of amphetamines. The basicity, tissue distribution and metabolism of β-fluoro-, β,β-difluoroamphetamine, and amphetamine were strikingly different in rats.[70] Nmr studies of the conformational properties[71] of amphetamine and related phenethylamines revealed a preference for trans phenylamino rotomers in aqueous solution,[72] although substituent groups, steric and electronic factors influence rotomer populations.[73] A quantum mechanical study indicated the folded form of amphetamine is slightly favored.[72] Metabolism of amphetamine to an oxime and subsequent hydrolysis to benzyl methyl ketone was shown to constitute a route of deamination.[74] In a rabbit liver oxygenase system this conversion involves an intermediate α-hydroxyamine.[75]

Hypotheses, Biochemistry and Pharmacology - Of the various ideas about biochemical causes of affective disorders,[76,77] the "catecholamine (CA) hypothesis" is still the most frequently cited. According to this postulate depression may be related to a deficiency of CA (usually NE) at functionally important central CA receptors, while mania results from excess CA. Perhaps an equally plausible case can be made for a "serotonin hypothesis of affective disorders." Consistent with the CA hypothesis are the observations that when α-methyltyrosine, an inhibitor of CA biosynthesis, was given to 7 manic and 3 depressed patients under double blind conditions, 5 of the manic patients became less manic and the 3 depressed patients became more depressed.[78,79] However, attempts to overcome the postulated deficits of CA or serotonin (5-HT) by administration of the amino acid precursors, L-dopa, tryptophan, or 5-hydroxytryptophan- a strategy successful in the treatment of parkinsonism - have given generally disappointing results in depressed patients.[78,80,81] Even in large doses equivalent to those found to be effective in parkinsonism, L-dopa produced an antidepressive effect in only 4 of 16 patients. All of the patients who responded had retarded depressions; none of the 5 patients with agitated depression improved. Five depressed patients who had prior

histories of mania developed hypomania or mania without a reversal of depression. It is suggested that CA, particularly dopamine (DA), may be involved in the genesis of mania in susceptible individuals but that depression and mania are not opposite poles of the same entity.[80] These results do not support the CA theory of depression but neither do they rule it out. Although L-dopa increases DA in brain, it may not increase NE. It also produces other central biochemical actions, e.g., effects suggesting that it causes a decrease in turnover of brain 5-HT in depressed patients.[82] However, the mostly negative clinical results with L-dopa seem to cast further doubt on the validity of the behavioral syndrome and amine depletion induced by reserpine (which is reversed by L-dopa) as a model of depression.

The catechol theory of depression is based in part on evidence that the tricyclic antidepressives and amphetamine affect CA metabolism in ways that result in an increased availability of the transmitters at their receptor sites. It has been thought for some time that the well known inhibitory action of the tricyclic antidepressives upon the uptake of NE in central noradrenergic (NA) neurons results in an increase of the monoamine at the receptors and may account for antidepressive activity. Several observations may not be in accord with this view. Indirect evidence has been adduced that some tricyclic agents with tertiary amino groups (imipramine, chlorimipramine and amitriptyline) preferentially block 5-HT uptake in 5-HT neurons while certain secondary amines (desmethylimipramine, nortriptyline and protriptyline) preferentially inhibit uptake of NE. Recent studies in which *in vivo* uptake of NE and 5-HT was directly measured histochemically confirm the earlier data as did functional studies using the extensor and flexor reflexes to assess the influence of the various drugs on 5-HT and NA receptor activity, respectively.[83] In addition the antihistamine drugs, chlorpheniramine and especially brompheniramine were found to be very potent blockers of 5-HT uptake. However, these drugs, unlike the tertiary amino tricyclics, also showed a potent blocking action on NE uptake. The questions arise whether the clinical activity of these drugs is more closely related to effects on 5-HT or on NE neurons or whether there are two classes of antidepressives acting by different mechanisms. Some clinicians feel that the clinical actions of the tricyclic antidepressives can be divided into a mood-elevating component and a psychomotor-activating component. They believe the tertiary amines are more effective in brightening of mood, suggesting involvement of 5-HT neurons while the secondary amines are more effective in increasing drive or producing psychomotor activation, suggesting that NE neurons are mainly involved.[83,84]

Seemingly at odds with the blockade of amine-uptake hypotheses are the properties of one of the newer tricyclics, iprindole. This drug produces clinical effects resembling those of imipramine-like agents yet in some pharmacological tests, e.g., reversal of reserpine-induced ptosis and hypothermia in mice, it is considerably less potent than imipramine.[85] It is a very weak inhibitor of uptake of NE and 5-HT *in vitro* and *in vivo* in mice and rats.[86] Despite its weak uptake blocking activity, it potentiated the awakening effect of L-dopa in reserpinized mice as

imipramine does. Unlike the antidepressives which block amine uptake, iprindole does not augment the pressor response to NE or block the blood pressure response to tyramine in man.[87]

Differences in the effects of acute and chronic treatment with imipramine and protriptyline on central NE metabolism in the rat have been observed.[88] The turnover of NE was decreased after acute administration but increased during chronic administration of these drugs. The increase in NE turnover occurred sooner when thyroxine was administered with imipramine. It is suggested that these observations may help to explain the delayed onset of clinical antidepressive effects and the accelerating and enhancing effects of thyroid hormone on the clinical effects of imipramine. It has been suggested that thyroxine may exert some of its action by facilitating the action of NE in central NA pathways.[89] Rats treated with thyroxine became hyperactive and showed increased sensitivity to the behaviorally activating effects of NE administered intraventricularly. Possibly relevant to the variation in effects on NE turnover noted under varied conditions of administration of the tricyclics is the observation that spontaneous activity in rats was decreased shortly after administration of imipramine but was increased over that of control animals one day after administration.[90] Much more data of the kinds cited above must be obtained from studies of a variety of tricyclic antidepressives, including iprindole, before any conclusions about a relationship between the clinical action of these drugs and their effects on monoamine metabolism can be drawn.

A study of the effects of L-dopa and Li_2CO_3 on urinary excretion of cyclic AMP in depression and mania has been reported.[91] Manic patients excreted the most cyclic AMP and severely depressed patients excreted the least; controls and moderately depressed patients showed intermediate values. Depressed patients treated with L-dopa showed marked increases in urinary cyclic AMP excretion. Patients treated with Li_2CO_3 showed changes in cyclic AMP excretion in the direction of clinical change; as depression improved, cyclic AMP excretion increased and as mania improved it diminished. It is not possible to attribute these changes in excretion of cyclic AMP to either central or peripheral mechanisms or both.

Rubidium ion seems to have opposite effects from lithium ion on CA metabolism and it has been suggested that it may have clinical antidepressive activity. It produces an increase in the release and turnover of brain stem NE and, unlike lithium ion, it induces an increase instead of a decrease in shock-elicited aggression in rats.[92] Further comparisons of rubidium and lithium ions may shed considerable light on the biochemistry of the affective disorders if the initial clinical observations that rubidium has antidepressive activity are confirmed by controlled studies.[28]

Although the roles of NE and 5-HT in the antidepressive action of the tricyclics have not been established, there is little doubt but that the stimulatory action of amphetamine is to a large extent mediated through CA pathways. Early experiments emphasized effects of the drug on NA systems. Now the evidence is stronger for a DA mechanism of central

action than for a NA mechanism. Although there is some disagreement, most authors believe that amphetamine produces its central effects by releasing dopamine and NE at nerve endings from labile intraneuronal pools of newly-synthesized amines. Amphetamine is thought to induce stereotyped behavior by releasing DA whereas apomorphine produces this behavioral effect by a direct action on DA receptors. The increase in locomotor activity elicited by amphetamine appears to be mediated at least in part by a NA action. Other behavioral effects of amphetamine may also involve NA and/or DA actions. The experimental basis for these statements has been extensively reviewed.[55,84] A few of the observations may be briefly summarized as follows. Depletion of CA in the intraneuronal storage granules by reserpine does not prevent the increase in motor activity or stereotypical behavior produced by amphetamine or apomorphine. Inhibition of CA synthesis by α-methyltyrosine blocks the effects of amphetamine but not those of apomorphine. Inhibitors of dopamine β-hydroxylase (e.g., diethyl dithiocarbamate), which inhibit the synthesis of NE but not DA, decrease amphetamine-stimulated motor activity, but do not inhibit and even may enhance amphetamine-induced stereotypies. Recent studies confirm these results.[93,94,95]

Most evidence suggests that amphetamine- and apomorphine-induced stereotypies are the result of enhanced DA activity in the neostriatum. Consistent with this view is the effect of amphetamine in rats with unilateral lesions of the nigrostriatal DA pathway (see Chapter 1, p 12). The drug causes ipsilateral turning in these animals, presumably as a consequence of release of DA in the neostriatum from the intact DA neurons of the contralateral (non-lesioned) side. This effect of amphetamine is attenuated by pretreatment with α-methyltyrosine but not with reserpine.[96,97] In contrast, apomorphine, which is believed to be a direct acting DA agent, induces contralateral turning behavior, an effect attributed to denervation supersensitivity of striatal DA receptors in the neostriatum on the lesioned side. This effect of apomorphine is not antagonized by either reserpine or α-methyltyrosine. In rats with unilateral lesions in the neostriatum, amphetamine and apomorphine both elicit ipsilateral turning. Similar effects are observed in mice with unilateral lesions of the caudate nucleus. This preparation provides a simple and rapid model for studying effects of drugs on central DA systems.[98]

Possibly at odds with the hypothesis that drug-induced stereotyped behavior involves a DA mechanism are observations on the effects of some stimulants on the turnover of brain CA in the rat. d-Amphetamine, aminorex and p-chloramphetamine at doses which increased motor activity, but did not induce stereotypies, caused an increase in turnover of DA but not of NE. These results are difficult to interpret, however, because phenmetrazine did not alter turnover of DA or NE at a dose that increased motor activity.[99]

Although the central actions of amphetamine may require an uninterrupted synthesis of CA rather than reserpine-sensitive stores of the amines, this does not seem to be true for all amphetamine-like stimulants.

A recent study, together with earlier data, suggests that amphetamine-like stimulants can be divided into two groups according to the ways in which they interact with reserpine and α-methyltyrosine. The effects of members of one group - amphetamine, methamphetamine, ephedrine, phenmetrazine and phentermine - are inhibited by α-methyltyrosine but not by reserpine; those of members of the second group - benzphetamine, methylphenidate, pipradrol, cocaine and amfonelic acid (7-benzyl-1-ethyl-1,4-dihydro-4-oxo-1,8-naphthyridine-3-carboxylic acid) - are inhibited by reserpine but not by α-methyltyrosine, at least in moderate doses.[100] Although, as these results suggest, stimulants may differ in their actions on intraneuronal pools of CA, no difference in their clinical effects has been observed. Methylphenidate produced physiologic, subjective and behavior effects that were not significantly different from those of amphetamine, methamphetamine, ephedrine and phenmetrazine.[101]

Further studies of the metabolism of amphetamine and of the effects of the drug and its metabolites on CA metabolism have not defined the biochemical effects underlying tolerance development to the drug.[102-104] A thorough study was made of the psychomotor and anorexigenic effects of d-amphetamine upon chronic dosing.[105] Tolerance development to the drug occurred in rats which were dosed daily shortly before testing. Tolerance did not occur in rats which received the same daily doses of amphetamine but were dosed after each daily test session. Subsequent dosing of the latter group with amphetamine prior to testing produced psychomotor stimulation and anorexigenic effects as large as those initially observed in the rats that had always been dosed daily prior to testing. The results suggest that tolerance to these effects of amphetamine represents a behavioral adaptation of the rats to the "drugged" state.

Several reports described effects of the methylxanthines and amantadine on brain monoamine metabolism which may be involved in their pharmacological actions. Caffeine and theophylline release brain NE in rats and guinea pigs.[106] The increase in cyclic AMP produced by these drugs, usually attributed to inhibition of phosphodiesterase, could be due to stimulation of adenyl cyclase by the released NE. In mice caffeine and aminophylline appear to increase turnover of brain DA and NE.[107] Other data suggest that the methylxanthines either prevent the release of brain 5-HT or increase 5-HT synthesis.[108] The antiparkinsonism effects of amantadine have stimulated studies of its central actions. Extensive biochemical and pharmacological data from studies in rats and mice indicate that this drug produces its effects, e.g. increase in rotational behavior in the rat turning model and increase in motor activity, by releasing CA, especially DA, from extragranular (reserpine-resistant) stores. Thus amantadine appears to have an amphetamine-like action but it is much less potent than amphetamine.[109,110] However, unlike amphetamine, amantadine-induced increase in motor activity in mice was not altered by α-methyltyrosine.[111]

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Chapter 3. Analgesics and Narcotic Antagonists

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Literature reports over the last year indicate some decrease in the amount of chemical effort devoted to new analgesic structures, but a continued increase in investigation of biochemical mechanisms of analgesia and dependence. Interest in narcotic antagonists for the treatment and prevention of narcotic abuse has stimulated much current research on new compounds. In neither area was there evidence of a significant advance during the year.

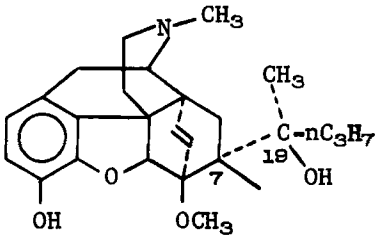
An extensive review of the literature on narcotic antagonists and analgesics from July, 1966 to April, 1970 appeared.[1]

I. Strong Analgesics

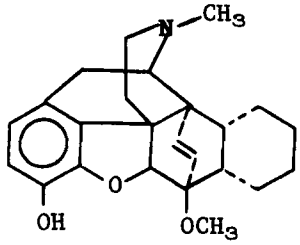
A. New Clinical Studies - No clinical studies on new compounds were reported, but new studies on diviminol,[2] tilidine,[3] and bezitramide[4,5] continue to indicate that these are orally effective analgesics in the codeine-morphine potency range which do not produce tolerance.

B. Structure-Activity Studies - There is an increasing tendency to use the newer tests (inflammed foot, writhing, bradykin) for evaluation of analgesic potency but, unless otherwise specified, potency estimates in the following discussion are based on the mouse hot plate assay.

1. Compounds Related to Morphine - New reports of the chemistry and activities of ethenothebaines and oripavines include a collated review of structure-activity relationships containing previously unreported data.[1a] The importance of an R configuration at C_{19} for high analgesic activity has been established[6] for the 7-β as well as the 7-α series. The primary



I

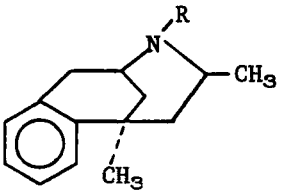


II

importance of a lipophilic binding site rather than that previously postulated for the C-19 hydroxyl was shown by the finding that II is as potent (1000 times morphine in the rat tail pressure test) as etorphine (I). Structure-activity relationships in this series vary greatly according to the test used.[1a]

Both natural (-) and synthetic (±) forms of the cholinesterase inhibitor, galanthamine, were reported to be analgesics equipotent with morphine.[7]

2. Benzmorphans - Synthesis and conformation of the 3- (III) and 4-methyl (IV) benzmorphans were reported.[8] IVa (2 times codeine) was about three times as potent an analgesic as IIIa, but IVb, c and d surprisingly were



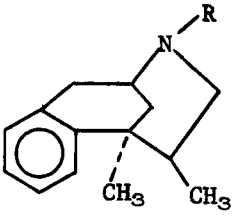
R = (a) $-CH_3$

(b) $-CH_2CH_2C_6H_5$

(c) $-CH_2$-cyclopropyl

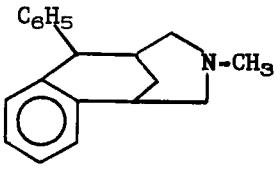
(d) $-CH_2CH=C(CH_3)_2$

III



IV

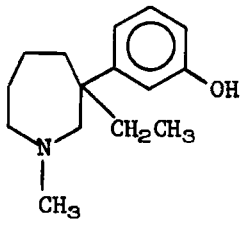
inactive. In the III series, all were active - the cyclopropylmethyl analog (IIIc) about five times as active as the methyl (IIIa) analog and without antagonist effect or physical dependence capacity in the monkey.[9]



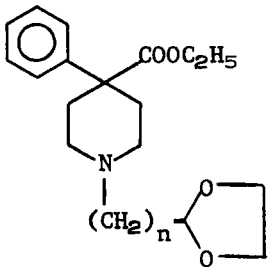
V

The benzmorphan analog (V) was comparable to codeine in the acetic acid writing test, but eleven other analogs oxygenated at the carbon bearing the phenyl group were inactive.[10]

3. Meperidine Analogs - WY-22811 (VI)[11] and both of its optical isomers were somewhat more potent analgesics than pentazocine, and both antagonized morphine, the (-) isomer being the more potent. Side effects were minimal.



VI

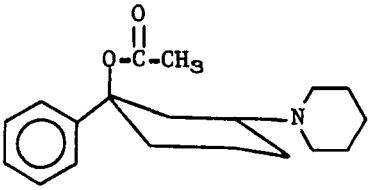


(a) n = 1

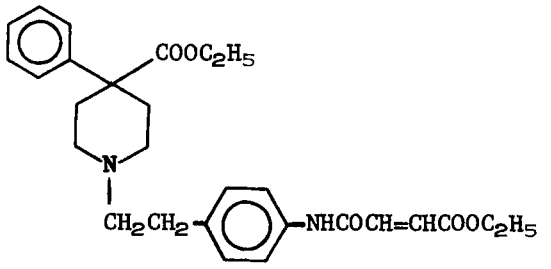
(b) n = 2

VII

The two dioxolanoalkyl meperidines (VII) showed activity in the meperidine range (VIIb was 3 times VIIa).[12] The corresponding dioxane analogs were inactive at 50 mg./kg. but did potentiate meperidine.



VIII



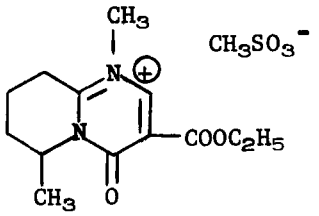
IX

The trans isomer of VIII showed weak analgesic activity while the cis was inactive, although it more closely approximates the structure of morphine than does the trans.[13]

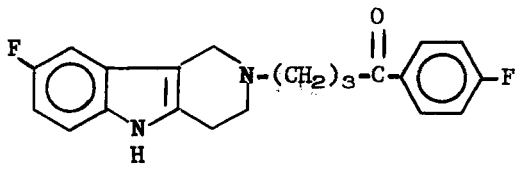
A series of anileridine derivatives bearing substituents with potential alkylating ability was made in order to investigate the possibility of locating analgesic receptors by specific alkylation.[14] The fumaryl derivative (IX) caused apparent inactivation which could be prevented by naloxone pretreatment. Other analogs of IX were non-selective and toxic.

An attempt to label receptors in brain homogenates by photolysis with [H^3]-N-β-(p-azidophenyl)-ethylnorlevorphanol also showed insufficient specificity although the compound was a potent narcotic.[15]

C. New Compounds - Extensive pharmacological studies of MZ-144 (X) and limited data on 20 analogs (of 85 prepared) were reported.[16-19] It was



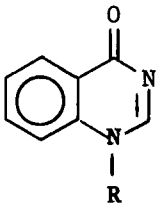
X



XI

orally active, did not produce tolerance, was not antagonized by nalorphine and strongly potentiated morphine even in tolerant animals. At high doses, MZ-144 was nearly as effective as morphine in animal studies, and it was said to be clinically effective at doses of 0.15-0.3 g.

Abbott 30360 (XI) was found to be an orally active analgesic-tranquilizer whose analgesic effect was in the morphine range.[20] Limited data on 14 analogs indicated fairly high structural specificity for analgesic potency, although the 8-CN analog was equally active.



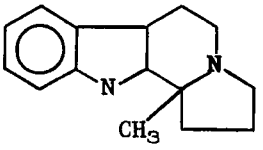
R = (a) $-CH_2CH=CH_2$

(b) $-CH_2C\equiv CH$

(c) $-CH_2CH=CHCl$

(d) $-CH_2$-cyclopropyl

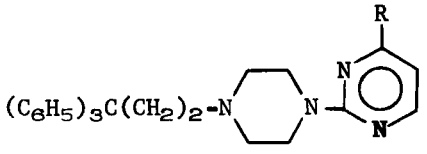
XII



XIII

Four quinazolones (XII) of a series of 19 were more potent analgesics than codeine. Substitution in the ring or modification of the R-group reduced activity. XIIa has been selected for clinical trial.[21]

WY-12157 (XIII) showed analgesic activity of the order of morphine as did both of its optical isomers.[22] It was not antagonized by naloxone and produced little or no tolerance, but CNS toxicity and blindness were seen during chronic administration to monkeys.[23]



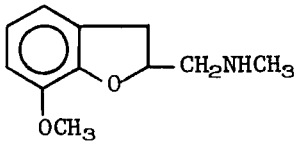
XIV

R = (a) -H

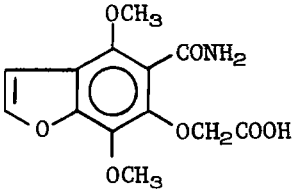
(b) $-NHCH_2CH=CH_2$

(c) $-NHCH_2$-cyclopropyl

XIVa was found to be a centrally-acting analgesic more potent than codeine with little respiratory depressant action or physical dependence capacity.[24] Twenty of 64 analogs made were also active. XIVb and c antagonized XIVa but potentiated morphine.



XV



XVI

XV[25] and XVI[26] were reported to have significant analgesic activity, but data were limited.

II. Antiinflammatory Analgesics

For complete coverage of this class of compounds refer to the chapter on Antiinflammatory Agents (Section 4). No new compounds with outstanding analgesic activity have appeared.

Reports on new clinical studies[27] on clonixin show equivalence of 600 mg. p.o. with 10 mg. of morphine parenterally in post-operative pain.

III. Biochemical Mechanisms

Many new data concerning the role of neurohumoral agents in the actions of analgesics continue to appear.

The conflict between different workers on the effect of morphine on brain serotonin turnover[28,29] may be due to different time courses of the experiments, since it appears to be an acute effect.[30]

Evidence has been reported suggesting that the role of catecholamines in analgesia may depend mainly on dopamine or dopamine/norepinephrine ratios.[31,32] Strong cross tolerance between the analgesic effects of methamphetamine and morphine develops after 5 days' administration.[33]

Additional studies on the depression of brain acetylcholine release by analgesics[34] have shown that morphine also facilitates its action at the neuromuscular junction.[35]

A review of knowledge concerning the pharmacokinetics and sites of action of narcotics in the brain appeared.[36]

IV. Narcotic Antagonists

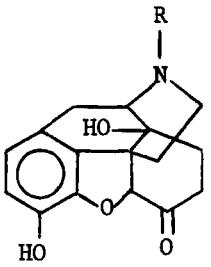
Over the past ten years, research on narcotic antagonists has been directed principally toward the discovery of new analgesics, since it was originally thought that such compounds would not have abuse liability. Work leading to the introduction of pentazocine has been discussed in a number of reviews.[1, 37-40] Structurally, these antagonists were polycyclic potent analgesic molecules with the N-methyl group replaced by an allyl, cyclopropylmethyl or related moiety. The most potent analgesic-antagonists did not attain practical utility because of hallucinogenic or other bizarre central nervous system effects that are usually associated with powerful antagonist activity.

More recent research on analgesics has produced new types of compounds that will not support morphine dependence, and some of which pharmacologically behave as mild morphine antagonists. These are not related structurally to the antagonists mentioned above. Direct addiction studies on both types of antagonists demonstrated a capacity to produce physical dependence (e.g. pentazocine,[41] nalbuphine,[42] profadol[43] and propiram[43]) of a type which could lead to drug seeking behavior.[44] (The potent antagonists with hallucinogenic effects can also produce a milder type of physical dependence not leading to drug seeking behavior.) Thus, it has been established that a component of antagonist activity does not necessarily eliminate abuse liability.

Two potent antagonists, naloxone (XVIIa) and cyclazocine (XVIII) were administered to selected groups of narcotic addicts in an attempt to "cure" their addiction by blocking any pleasurable effect of narcotic self-administration. The results[45,46] of the series of preliminary studies were sufficiently favorable that large-scale controlled studies were begun to better evaluate this type of approach. Research in this

area is being expanded greatly as part of the effort[47] now being directed toward alleviation of the drug abuse problem. Although some of the current research in this field has been discussed in newspapers and magazines, few data suitable for this review have been published. Data reported so far indicate that naloxone has too low an oral potency and too short a duration of action to be satisfactory, while the side effects of cyclazocine may be unacceptable.

Naloxone (XVIIa) is the only reported narcotic antagonist without other biological activity.[48] This can be demonstrated by an acetylcholine release assay[49] which measures both agonist and antagonist actions of narcotics, as well as by pharmacological studies. In contrast, analogs

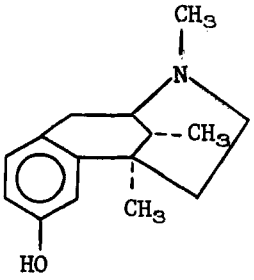


R = (a) $-CH_2CH=CH_2$

(b) $-CH_2$-cyclopropyl

(c) $-CH_2$-cyclobutyl

(d) $-CH_2CH=C(CH_3)_2$

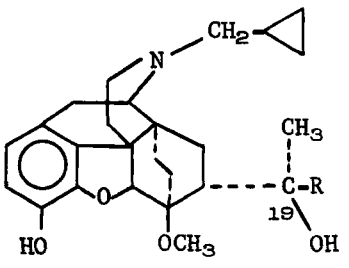


XVII XVIII

XVIIb, c and d show both analgesic and antagonist properties. XVIIb (EN-1639) is currently being studied in addicts[50] since it appears to be longer acting than naloxone, and shows fewer side effects than cyclazocine.

Cyclazocine (XVIII) is a powerful antagonist and analgesic, and its many known analogs show both properties.

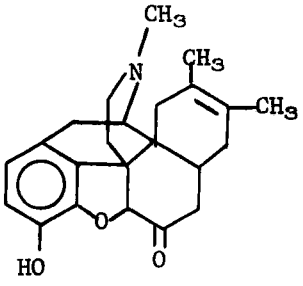
Diprenorphine (XIXa) is a pure morphine antagonist (3-5 times naloxone) pharmacologically, but shows some agonist activity in the acetylcholine assay.[51] In this series, structure around C-19 is critical, e.g. XIXb and c are not antagonists.[52]



R = (a) $-CH_3$

(b) $-C_3H_7$

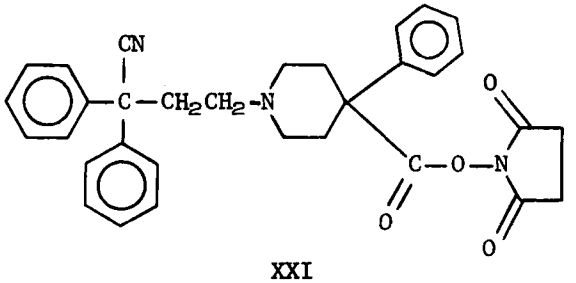
(c) $-C_4H_9$



XIX XX

XX shows mild antagonist activity which is unusual for an N-methyl compound.[1a]

Diphenoxylate and an analog SC-26100 (XXI) will prevent withdrawal symptoms in morphine-addicted animals, and the former has been reported to have the same effect in man.[53]



XXI

Haloperidol has been reported to be useful in the treatment of withdrawal symptoms in addicts and also to reduce morphine self-administration in rats at the proper dose.[54]

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Chapter 4. Sedatives, Hypnotics, Anticonvulsants and General Anesthetics

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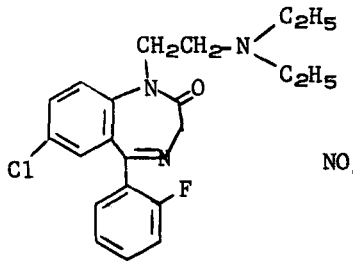
Introduction - Although an effort has been made in this chapter to exclude depressants classified in other chapters as antipsychotic and antianxiety agents, certain benzodiazepines will be considered here because of the number of compounds in this series which have been found clinically to possess sedative-hypnotic and anticonvulsant activity. In most cases, compounds with more than preliminary pharmacological data or representatives of new structural types were selected for inclusion in this chapter.

Sedatives and Hypnotics - Considerable research effort has been expended to elucidate the possible role of serotonin in the neuropharmacology of sleep[1]. Administration of serotonin in proper dosage and by the proper route produces electroencephalographic and ocular signs of (slow wave) sleep. Nonspecific reduction of serotonin levels in the brain by reserpine reduced both rapid eye movement (REM) and non-rapid eye movement (NREM) sleep while sterotaxic destruction of serotonin-containing neurons has produced insomnia in animals.[2] With the discovery of a specific inhibitor of serotonin synthesis, para-chlorophenylalanine, it has been possible to deplete brain serotonin without altering the catecholamine content markedly[3]. A dose dependent reduction in cerebral serotonin, induced by the administration of para-chlorophenylalanine, is followed by a dose-dependent reduction in total sleep lasting as long as 16 days.[4] Injection of 5-hydroxytryptophan at the height of the para-chlorophenylalanine action restored slow wave sleep within 10 minutes and after a delay of 5 hours, paradoxical sleep. In man, para-chlorophenylalanine decreased rapid eye movement (REM) sleep, with either no change or a slight increase in non-rapid eye movement (NREM) sleep[5].

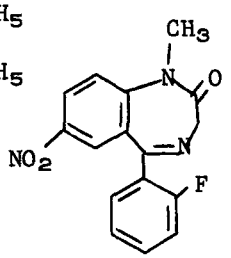
L-Tryptophan, a precursor of serotonin has also been shown in normal subjects to reduce sleep latency and increase sleep length without altering the qualitative characteristics of polygraphically recorded sleep[6]. In a double-blind study in hospitalized insomniac patients, doses of 4 to 5 g of L-tryptophan significantly increased sleep time, reduced sleep latency and reduced the number of awakenings.[6]

Several reviews have recently appeared on the neurohumoral aspects of sleep control[4] and on the chemical families which influence sleep patterns.[7]

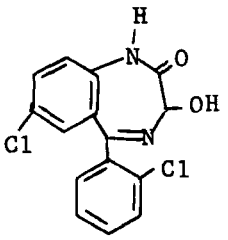
Many benzodiazepine derivatives have been reported to possess sedative and hypnotic activity in both animals and man. Flurazepam (I) has been found to possess useful hypnotic activity in man without producing significant "hangover" effects the following morning[8,9]. In animals flurazepam was found to possess activity, in many tests systems, similar in potency and profile to diazepam[10].



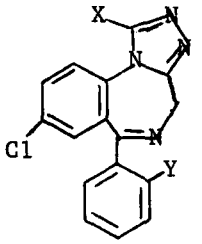
I



II



III

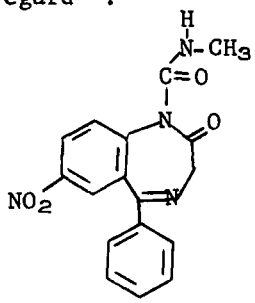


IV-X=H Y=H
V-X=CH_3 Y=H
VI-X=CH_3 Y=Cl

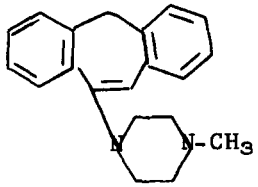
Flunidazepam (II) was found in all-night human EEG sleep studies and other efficacy studies to be a potent hypnotic approximately 15 to 30 times more active than flurazepam [11,12]. Recently an analog of oxazepam , lorazepam (III, WY-4036) was reported by Hedges et al [13] to possess potent sedative and depressant activity in man and was approximately 5 times more active than diazepam in this study.

Perhaps the most significant benzodiazepines reported in the last two years have been the triazolobenzodiazepines (IV, V, VI). These compounds have been found to possess sedative and hypnotic activity in animals [14,15,16] and in man [17]. From experimental data in man, Compound V was predicted to have long lasting antianxiety properties at low doses and sedative-hypnotic activity at high doses [17].

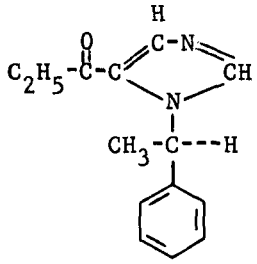
Another benzodiazepine derivative, VII, has been reported to possess hypnotic activity in animals and is more potent than diazepam in this regard [18].



VII



VIII

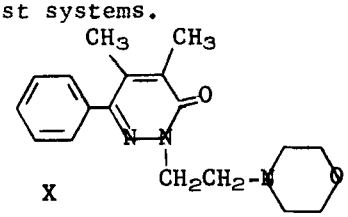


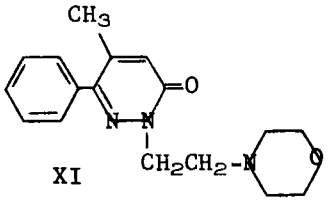
IX

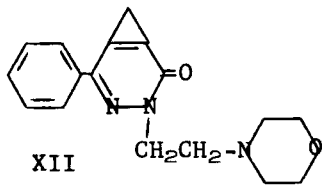
While the most significant sedatives and hypnotics found recently have been benzodiazepine derivatives, several interesting non-benzodiazepine derivatives have also been reported to possess sedative and hypnotic activity. One of these compounds, perlapine (VIII), has been found to be a useful hypnotic in man.[19]

Janssen et al[20] has reported that etomidate (IX, R-16659) is a potent i.v. hypnotic in rats with a rapid onset of action and a duration of hypnosis dependent on the dose administered. The compound has a high safety margin with the toxic dose being approximately 30 times the efficacious dose.

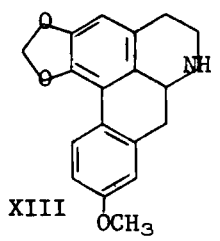
Three new pyridazone derivatives (X, XI, XII) were reported by Wermuth et al[21] to possess sedative and hypnotic activity. The compounds were synthesized in an analgesic program but were poorly active in analgesic test systems.

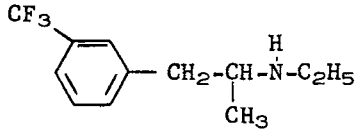

X

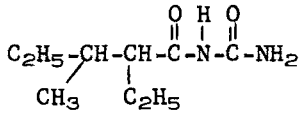

XI


XII

An interesting aporphine alkaloid, xylopine (XIII), isolated from the bark of Xylopia brasilienes, possesses both sedative and analgesic activity in mice at moderate doses[22]. In dogs the compound was found to possess hypotensive activity.


XIII


XIV


XV

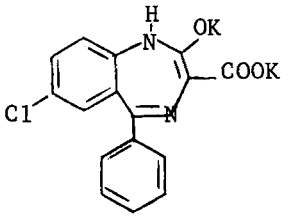
In contrast to many other classes of compounds, the activity of many CNS agents has been found clinically.Fenfluramine is an example of such a clinical finding. Fenfluramine (XIV) is presently marketed in 22 countries as an effective and safe anorexigenic drug. Though a phenethylamine derivative, the compound produced sedation and drowsiness in man as a side effect in the anorexigenic studies[23].

A ureide derivative, capuride (XV, McN-X-94) is a new addition to the nonbarbiturate sedatives. It is especially useful in aiding sleep in preoperative patients[24].

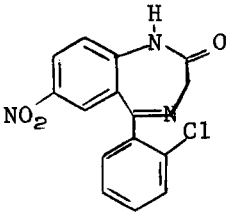
Finally, a new derivative of cyclic 3'5'-adenosine monophosphate, HD-233(6-piperidino-purinoribosyl-3'5'cyclic AMP) has been found to have a sedative effect in rats[25]. The compound does not alter either serotonin or 5-hydroxy-indoleacetic acid levels in the brain of rats but does increase the brain levels of barbiturates and prolongs barbiturate sleeping time.

Anticonvulsants - During the past two years numerous compounds possessing anticonvulsant activity have been reported. Many of the compounds report-

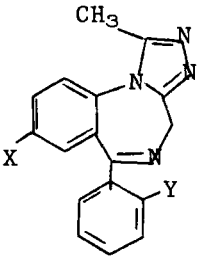
ed were benzodiazepine derivatives and these will be reviewed briefly in this section. Chlorazepate (XVI, A-35616, 4306CB) possesses anticonvulsant activity in mice equipotent to diazepam[26]. The monopotassium and the dipotassium salts possessed approximately the same activity and have the advantage of being water soluble. Although the pharmacology of clonazepam (XVII, Ro 5-4023) has been reported previously in this review, more recently numerous papers have appeared reporting the potent anticonvulsant activity in animals[27] and man[28,29]. Clonazepam has been found to be 10 times more active than diazepam.



XVI



XVII

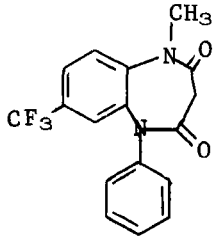


XVIII-X=Cl Y=Cl
XIX- X=Cl Y=H
XX- X=H Y=H
XXI- X=H Y=Cl

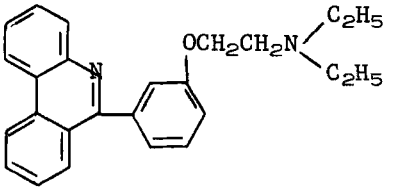
A series of triazolobenzodiazepines were found to possess potent anticonvulsant activity in animals. U-33,030 (XVIII) and U-31,889 (XIX) were much more potent than diazepam in mice, rats and cats while U-31,957 (XX) and U-35,005 (XXI) were equipotent to diazepam[15,16,30]. These compounds are extremely active against chemically induced seizures but only weakly active against seizures induced by electroshock.

Removal of the chloro-group from position 7 in the diazepam molecule markedly decreased the pharmacologic activity while in the triazolobenzodiazepine series the compound (XX) still was equipotent to diazepam. This points out the major differences in the triazolobenzodiazepine structure activity relationships compared to the diazepam series.

Another benzodiazepine derivative possessing anticonvulsant activity is ORF-8063 (XXII). The compound inhibits maximal electroshock, pentylenetetrazol and thiosemicarbazide convulsions in laboratory animals[31].



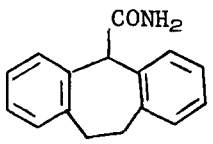
XXII



XXIII

XXIV

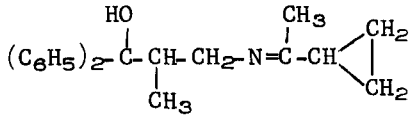
A series of 22 phenanthridine basic ethers reported by Ashford et al were found to possess anticonvulsant activity in mice[32]. Some of the derivatives, of which compound XXIII is a representative, were as active as diphenylhydantoin in antagonizing pentylenetetrazol and electroshock convulsions but in most cases possessed therapeutic ratios inferior to diphenylhydantoin.



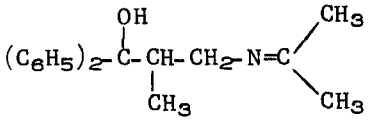
XXV

Funcke and Zandberg, evaluating a series of tricyclic compounds for anticonvulsant activity, found two compounds, BS7679 (XXIV) and BS7029 (XXV), worthy of further study[33]. The compounds were more efficacious than phenobarbital with a low neurotoxicity.

A series of diphenylaminopropanols were evaluated for toxicity, anticonvulsant, anorexigenic and anticholinergic activity[34]. Several of the derivatives (XXVI, XXVII) were potent anticonvulsants against thiosemicarbazide, electroshock and nicotine induced convulsions without possessing anticholinergic activity.

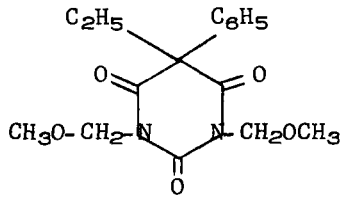


XXVI

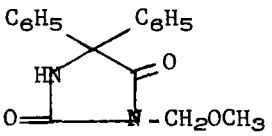


XXVII

Alkoxymethyl derivatives of barbiturates and diphenylhydantoin are effective anticonvulsants[35]. The compounds (XXVIII, XXIX) possess marked activity against both maximal electroshock and pentylenetetrazol induced seizures.



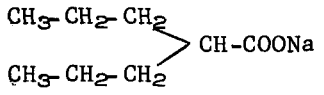
XXVIII



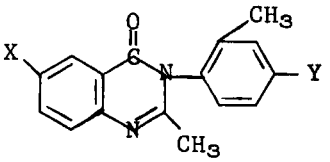
XXIX

The same authors also reported that acyloxymethyl, bis(acyloxymethyl) and bis(halomethyl) derivatives of 5-ethyl-5-phenylbarbituric acid possessed marked anticonvulsant activity[36].

Depakine (XXX) has been reported by DeBiolley and Sorel in uncontrolled studies to be an effective anticonvulsant in the treatment of petit mal epilepsy[37,38]. Some action was also noted in psychomotor seizures. Side effects in these studies were not a major problem.



XXX

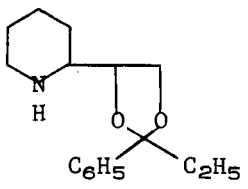


XXXI - X=H Y=NH_2
XXXII - X=NH_2 Y=Cl

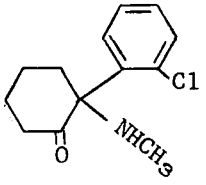
Finally, various compounds related to methaqualone have been found to possess anticonvulsant activity in mice[38]. Compound XXXI was found to be more active than methaqualone against maximal electroshock seizures. Compound XXXII also possessed potent anticonvulsant activity but was more toxic than Compound XXXI.

General Anesthetics - Few new anesthetic agents were reported in the literature in the last two years. The pharmacology of a new dissociative anesthetic, etoxadrol (XXXIII, CL-1848C, U-37,862A) in primates and other species[39] and an evaluation of etoxadrol in normal human volunteers[40] has been reported. Etoxadrol has properties similar to ketamine (XXXIV) as an anesthetic. After intravenous administration in monkeys etoxadrol produces complete analgesia with retention of the light and corneal reflexes. In man, etoxadrol appears to be a useful drug in anesthesia and has a longer lasting activity than ketamine. Etoxadrol appears to be devoid of long term psychologic, biochemical or physiologic pathology.

Further studies have been conducted on compound 347 (XXXV), now called Ethrane® The compound was administered to 250 patients as the major anesthetic[41]. Its physical properties and clinical characteristics are similar to those of halothane.



XXXIII



XXXIV

HCF_2OCF_2CHFCl

XXXV

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Chapter 5. Recent Developments Relating Serotonin and Behavior

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The existing encyclopedic reviews on 5-HT[1] make evident that techniques for modifying 5-HT kinetics are almost as varied and voluminously reported as those for studying behavior. As a result, few areas are more difficult to survey briefly than this, relating two such ill-defined yet exhaustively studied variables. We have accordingly chosen to focus on a limited number of issues with particular current importance.

Techniques for Manipulating 5-HT Steady State Levels and Receptor Activity

It must be asserted at the outset that there are still no entirely satisfactory means to either elevate or reduce 5-HT steady state levels. Studies purporting to show altered behavior as a function of variations in 5-HT levels are inevitably tainted by factors that covary. For example, the elevation of brain 5-HT levels by administration of the dietary 5-HT precursor, Trp, leads also to the metabolism of excess substrate through other pathways, particularly the kynurenine pathway. Administration of the immediate precursor of 5-HT, 5-HTP, leads to a non-physiological distribution of 5-HT in brain, owing to the ubiquitous distribution of aromatic amino acid decarboxylase.[2] 5-HT derived from either precursor may decrease catecholamine stores by amine displacement, and Trp derivatives also inhibit tyrosine hydroxylase.[3] Conclusions based on high 5-HT levels in selected animal strains, at certain times of day, or at certain ages are confounded by multiple genetic, circadian or ontogenetic effects that covary. Similar problems apply to conclusions drawn from carcinoid victims or other examples of human pathology where 5-HT is elevated. MAO inhibitors, even those relatively specific for that isoenzyme for which 5-HT is a primary substrate, exert actions other than elevating 5-HT.

Comparable lack of specificity plagues known methods of depleting 5-HT from brain: reserpine and similar amine-releasing drugs block the intraneuronal vesicular uptake of catecholamines as well as of 5-HT.[4] Lesions in the nucleus Raphé, the medial forebrain bundle and other tracts,[5] which deplete 5-HT from brain, deplete NE as well. Prolonged Trp deficient diets[6] cause general nutritional deficits and an inefficient decrease in 5-HT levels. Substituted phenethylamine derivatives, such as p-chloromethamphetamine and fenfluramine, have usually been reported to exert relatively small effects on brain 5-HT and the mechanism

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Abbreviations used in this paper: 5-HT = 5-hydroxytryptamine (serotonin); Trp = tryptophan; 5-HTP = 5-hydroxytryptophan; 5-HIAA = 5-hydroxy-3-indoleacetic acid; MAO = monoamine oxidase; PCPA = p-chlorophenylalanine; NE = norepinephrine

of their action is unclear. The recent disclosure that intraventricularly administered 5,6-dihydroxytryptamine causes selective degeneration of 5-HT neurons in rat brain[7] is exciting, but will require confirmation.

The selectivity and activity problems inherent in the above techniques explain why PCPA[8] has been seized upon as a tool for studying behavioral and neurophysiological effects after brain 5-HT is depleted. PCPA is perhaps both the most selective and effective depletor of 5-HT known. Nevertheless, it also slightly, but significantly, depletes the brain of catecholamines for a few hours after its administration,[8] and several authors have dwelled on this effect in accounting for some of its physiological actions.[9] By blocking phenylalanine hydroxylase,[8,10] PCPA elevates phenylalanine and its metabolites[11] and "experimental PKU" may account for certain behavioral effects of PCPA. Other biochemical complications after PCPA include reduced uptake of Trp and phenylalanine.[12] Furthermore, 5-HT depletion after PCPA is not "complete", and residual amounts of 5-HT that are synthesized after PCPA may account for its unexpected effects or lack of effects.[13]

It is unnecessary to alter steady state levels of 5-HT in order to alter 5-HT receptor activity. Blockade of 5-HT reuptake into presynaptic terminals may prolong high extraneuronal concentrations of 5-HT.[14] Agents believed to exert such an effect -- tertiary tricyclic antidepressants, particularly chlorimipramine -- also have selectivity problems, since all exert at least some effect on NE uptake. Alternatively, it may be possible to stimulate or to block 5-HT receptors specifically. It is suggested that quipazine (2-[1-piperazinyl]quinoline)(III) exerts 5-HT mimetic properties based on its smooth muscle spasmogenic profile;[15a] its behavioral effects in animal tests also suggest similarities with 5-HT.[15b] From the plethora of agents exerting peripheral antiserotonin properties some may exert comparable effects in brain; the often-cited anti-5-HT activity of LSD in brain, however, may be complicated by 5-HT mimetic properties as well.[16]

Keeping specificity limitations in mind, let us nevertheless focus on the behavioral effects associated with a reduction in brain 5-HT levels caused by the tryptophan hydroxylase inhibitor PCPA or its methyl ester. Where possible the behavioral effects reported to occur after PCPA are related to those produced when 5-HT levels or receptor activity are altered by other means.

Behavioral Effects of PCPA-Elicited Serotonin Depletion in Animals

Symptoms - A corollary to the familiar Brodie and Shore hypothesis[17] that 5-HT mediates a central parasympathetic ("trophotropic") system is that suppression of 5-HT biosynthesis should result in central stimulation because of the dominance of the central sympathetic ("ergotropic") system. Initial symptomatological observations after PCPA, however, included only mild irritability and aggressiveness in isolated rats, without clear evidence of motor stimulation.[8] These observations of absent or mild symptomatic effects in rats have frequently been confirmed.[18] Quantitative

locomotor activity studies in rodents usually report no elevation, and sometimes decreases, in motor activity following PCPA at doses effective in depleting 5-HT.[19] Nevertheless, animals have exhibited elevated activity during prolonged exposure to an activity chamber, as if the novelty of the activity box is sustained.[20] In unrestrained larger animals, especially rabbits, cats and rhesus monkeys, irritability and aggressiveness without frank motoric excitement, are observed.[21]

Consummatory Behavior - Rats treated with moderate doses of PCPA consume laboratory food and water normally,[18b,19c,22] but appear to be more finicky or responsive than controls in their consumption of solutions of alcohol,[22,23] saccharin,[23c] dextrose[18b] or quinine,[18b] suggesting that gustatory or olfactory thresholds may be lowered by PCPA.

Sleep - Many sleep researchers have found that PCPA causes prolonged EEG activation and sleep disturbances. In rats, there is a significant decrease of both REM and slow wave sleep corresponding to reduced 5-HT levels in brain, but not with PCPA levels.[18a,20a,24] This diminution in REM sleep in rats is most unusual in that no REM rebound occurs.[24] Related to the sleep findings is the observation that PCPA inhibits hibernation in squirrels.[25] Extensive studies in the laboratories of Jouvet,[26] Koella,[27] Dement[21a] and others have shown that PCPA has the ability to block slow wave sleep and produce prolonged insomnia in cats, effects that are rapidly reversed by 5-HTP. Titration of PCPA and 5-HTP can lead to normal sleep. In rhesus monkeys depleted of brain 5-HT by PCPA, however, REM sleep may be unchanged, and in this respect PCPA effects appear to differ from those seen in rats and cats. The amount of time spent in slow wave (and hence, total) sleep decreases significantly, but "total insomnia", as often reported for cats, is not seen.[28] Dement et al,[21a] however, have been able to produce sleep loss in 2 monkeys resembling the effect in cats. To the extent that conclusions may be drawn from the data available, a different picture occurs in man. In patients seriously ill with carcinoid, Huntington's chorea, migraine, or dystonia, REM sleep is markedly depressed, but slow wave sleep is either unchanged or slightly increased.[29] A study of PCPA in normal volunteers revealed no subjective complaints of insomnia.[30]

The rat and cat sleep results with PCPA, even considered alone, would suggest that 5-HT is functionally involved in normal sleep mechanisms, but these findings are in fact only the latest in a long chain of circumstantial evidence linking 5-HT to sleep.[26b,27] Exogenous 5-HT, given intraventricularly or in the carotid, and 5-HT precursors -- either Trp or 5-HTP -- given peripherally, have frequently been reported to produce electrophysiological, behavioral and clinical evidence of sedation and sleep. Among other established means of reducing slow wave sleep are administration of reserpine and methysergide. Circadian variations in 5-HT brain levels in animals are positively correlated with circadian variations in sleep. Ablation of the 5-HT-rich Raphé nuclei also produces insomina.

Sensitivity to Pain and Shock Avoidance Behavior - PCPA is hyperalgesic

in rats,[19c,31] an effect reversible by 5-HTP, but not by 5-HT administration. 5-HT-depleting lesions of the medial forebrain bundle exert a similar effect.[31a] Heightened responsiveness to pain may account for enhanced avoidance acquisition after PCPA.[19c,32] It is also likely that elevations in avoidance response rate after PCPA[33] derive in part from increased sensitivity to foot shock.

PCPA blocks the analgesic actions of morphine, methadone, propoxyphene and meperidine.[34] CNS effects of morphine other than analgesia are blocked by PCPA,[35] but the findings of Way et al[36] that PCPA blocks symptoms accompanying morphine withdrawal have not been confirmed.[37]

The hyperalgesic and analgesic-blocking effects of PCPA are consistent with literature reports showing increased sensitivity to pain after lesion-induced depletion of 5-HT[31a] or after reserpine-like drugs, and an analgesic response following MAO inhibitors plus 5-HTP.[38]

Sensitivity to Convulsant Treatments - PCPA facilitates convulsions elicited by hyperbaric oxygen,[39] pentylenetetrazol,[40] electroconvulsive shock,[40a,41] audiogenic convulsant stimuli,[42] flurothyl[43] and withdrawal from barbiturates.[44] A substantial older literature, originating with the work of Chen et al[45] on reserpine, deals with the role of biogenic amines in experimental seizures. The preponderance of these reports indicate that stimulation of 5-HT receptor activity decreases seizure susceptibility, and that depression of 5-HT receptor activity exerts the opposite effect.

Sensitivity to Other Non-Social Exteroceptive Stimuli - Several studies indicate that PCPA increases various behavioral or neurological effects of electrical stimulation. Thresholds of current required to reinforce self-stimulation behavior are lowered by PCPA in rats,[46] and PCPA has been reported to cause strong excitatory effects on intracranial self-stimulation rates in rats 3 days after treatment.[47] 5-HT-depleted animals have been reported to be more active than controls under conditions of extra environmental stimulation, particularly "novel" stimulation.[18b,19c] Cats "overreact to slight noises,"[48] sensory thresholds are lowered,[29] the responsiveness to a startle stimulus of rats with low basal responsiveness is increased,[49] and habituation to an auditory-elicited startle response, as measured by cortical electrical responses, is reduced.[50]

Sexuality - Despite occasional dissent[51] there can now be little doubt that PCPA dramatically enhances sexuality in suitable animal protocols.[52] Like other effects of PCPA, hypersexuality is abruptly reversible by low doses of 5-HTP.[52a] PCPA has been observed to activate lordosis, a prime indicator of estrus behavior, in ovariectomized rats treated with estrogen and exposed to a vigorous male partner.[53] PCPA dramatically increases sexual behavior in cats[21a,54] and rabbits;[55] in these species, also, the effect of PCPA is reversed by 5-HTP.

Aggression and Other Non-sexual Social Behavior - Mouse killing (muricide) in rats is enhanced by PCPA.[22b] "Cats who normally ignore laboratory rats begin to kill them with a rapidity and concentrated savagery that

evoke images of the jungle".[21a] Mice or rats can be extremely aggressive to one another.[18a,22b,56] Much of this social behavior is reversed by administration of 5-HTP.[22b,52c]

Summary of Behavioral Effects of Depleting 5-HT with PCPA in Animals - Although isolated animals treated with PCPA show few symptomatic effects, exposure of animals to novel exteroceptive stimulation evokes behavioral hyperresponsiveness. Animals overreact to painful, electrical, auditory, visual, gustatory, olfactory and especially social stimuli. The sustained wakefulness of animals treated with PCPA may feasibly be considered one manifestation of this generalized hyperresponsiveness.

The animal behavioral data from PCPA, taken as a whole, may be loosely interpreted as suggesting that 5-HT subserves behavioral inhibitory functions, and that a relative absence of 5-HT produces hypersensitivity to environmental cues. Both the rapid reversal of electrophysiological and behavioral changes following small doses of 5-HTP, and the parallel time course of PCPA actions with brain 5-HT levels clearly associate 5-HT with its effects. The PCPA data are also consistent with findings from less selective means of modifying brain 5-HT, such as reserpine or MAO inhibitor treatment, treatment with 5-HT precursors, lesion-elicited 5-HT depletion, and the use of animal strains, ages, or times of day associated with variations in 5-HT.

Implications of PCPA-Elicited Clinical Symptomatology

Many clinical investigators who have studied altered attention, sensory and perceptual characteristics in man believe that schizophrenics respond to sensory inputs that do not affect normals.[57] In view of this, and the preceding analysis of the role of 5-HT in behavior, one might expect aberrant clinical behavior after selective depletion of 5-HT. Limited reports of such aberrations after PCPA have appeared.

In the course of successful trials of PCPA in patients with carcinoid tumor, pronounced behavioral aberrations, ranging from depression to florid hallucinations and paranoia occurred.[58] A comparison of sleep patterns in both schizophrenics and PCPA-treated cats revealed several similarities.[21a]

It thus appears at least feasible that the animal behavioral findings after PCPA are manifested in humans as depression and paranoia. This speculation is provocative since abnormalities of 5-HT synthesis and metabolism have been implicated in several psychopathological conditions. We shall accordingly now focus on the role of 5-HT in depression, mania, and schizophrenia and shall emphasize experimental therapeutic approaches - current and speculative - which involve an attempt to manipulate brain 5-HT.

Indoleamine Dysfunction in Human Behavior

Depression - The controversy surrounding the relative importance of catecholamines and indoleamines in depression has culminated in divergent

"catecholamine"[59] and "5-HT"[60] hypotheses. However, recent reviews on the role of brain monoamines in psychopathological disorders emphasize the need for understanding their complex interaction.[61]

Evidence cited to support 5-HT dysfunction in depression includes 1) decreased levels of 5-HT and 5-HIAA in the brains of depressed patients who commit suicide,[62] 2) sub-normal concentrations of 5-HT metabolites in the cerebrospinal fluid (CSF) of depressives,[63] and 3) diminished rates of 5-HT turnover, manifested as deficient 5-HIAA accumulation in the CSF following treatment with the efflux blocker, probenecid.[64]

The 5-HT precursors Trp and 5-HTP have been explored most extensively by proponents of the 5-HT deficiency hypothesis, in efforts to increase brain 5-HT levels.[65] The earliest trials of Trp in depression found it to potentiate the beneficial effects of MAO inhibitors.[66,67] Ensuing reports have claimed that D,L-Trp is as effective as electroconvulsive shock therapy[68] or imipramine[69] and it has been successfully used "as a last resort".[70] In other studies, however, L-Trp has been less impressive.[71]

Early studies of 5-HTP were limited to low doses given over short periods of time.[72] Recent, more prolonged trials, have yielded conflicting results.[73]

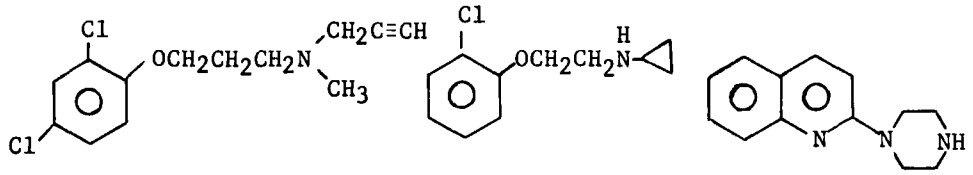
Several recent findings may have an important bearing on the future use of 5-HT precursors in depression. Evidence from CSF experiments suggests an abnormally low rate of 5-HT synthesis in the CNS of some depressed patients,[63] perhaps due to impaired Trp-5-hydroxylase activity.[74] If this were true, L-Trp would inefficiently elevate brain 5-HT in this population, explaining the divergent clinical results obtained with L-Trp. The use of L-5-HTP, which enters the 5-HT pathway subsequent to the rate-limiting Trp hydroxylation step, might seem a logical alternative. Recent reports, however, indicate that this precursor leads to indiscriminate and non-physiologic elevations of brain 5-HT.[2,75] To what extent these findings apply to the chronic administration of this amino acid to man is not known, but van Praag and Korf[73b] recently reported L-5-HTP to be effective in depressives who showed evidence of a 5-HT synthesis defect.

The continued viability of the 5-HT precursor approach to the treatment of depression is thus questionable. Additional definitive research concerning 1) the relationship of change in levels of 5-HIAA in CSF to brain 5-HT turnover, 2) the biochemical classifications of depression, and 3) the effects of chronic L-5-HTP on levels of physiologically active 5-HT are needed to determine the future direction of this approach.

Other potentially significant approaches to the treatment of depression based on the 5-HT hypothesis have emerged during the past few years. These include: 1) selective 5-HT reuptake blockade, 2) selective inhibition of MAO, and 3) 5-HT mimetic activity. Although these approaches may not suffer from the obvious disadvantages of the precursor approach, each possesses unique and challenging problems of its own.

The concept of selective blockade of monoamine reuptake has been studied extensively by Carlsson and associates[76] who have shown that tricylic amines such as imipramine and chlorimipramine are selective inhibitors of 5-HT uptake. Furthermore, they have suggested that blockade of 5-HT reuptake is associated with the "mood-elevating" action of the tricyclic antidepressants and that blockade of NE reuptake promotes "drive" in depressed patients. This is consistent with the fact that chlorimipramine, the most potent and selective inhibitor of 5-HT reuptake in this series, has encouraging antidepressant activity in humans[77] with at least one report of a selective mood-elevating effect.[78]

At least four different molecular forms of MAO, with different substrate specificities, occur in human brain[79] suggesting that depression might be treated by selective inhibition of 5-HT oxidation.[80] Several



I II III

selective inhibitors have been reported to date[81] and at least two, clorgyline[82] (I) and Lilly 51641 (II),[83] have been reported to possess antidepressant activity. It is not clear whether they possess sufficient selectivity to obviate the autonomic side effects commonly seen with other non-selective MAO inhibitors.

Another conceivable approach to the problem of 5-HT and depression is the development of an agent that selectively stimulates 5-HT receptors. Such a 5-HT mimetic would require a high degree of CNS selectivity, but this problem does not seem insuperable. Quipazine (III), a smooth muscle stimulant with 5-HT-mimetic properties,[15a] possesses a characteristic "antidepressant" profile in animals[15b] and, depending on clinical activity, may be a significant development toward this end.

Mania - Mania has been viewed historically as "the other affective disorder," and emphasis has tended to focus on the polarity of certain manic depressive symptoms. Kety[61c] has recently hypothesized that a central indoleamine deficiency permits the occurrence of either mania or depression, and that an accompanying change in adrenergic activity determines the polarity of the affective disorder, adrenergic activity being intensified in mania and diminished in depression. Thus, within the context of this discussion, Kety's hypothesis suggests a similar "pro-indole" therapeutic approach to both depression and mania.

Clinical trials of 5-HT precursors in mania have been rare, but two recent reports indicate a moderately beneficial effect from L-Trp.[71b,84] Methysergide has a deleterious effect in manic patients.[85]

Schizophrenia - Schizophrenia is almost certainly a class of behavioral disorders and not a single disease entity, which perhaps explains in part the numerous theories and conflicting clinical findings.[61]

Several theorists invoke defective indoleamine metabolism and transport as key etiological factors in schizophrenia.[61a,b] Woolley[86] suggested that 5-HT concentrations might be elevated in schizophrenics, consistent with observations that L-Trp in combination with an MAO inhibitor exacerbates psychoses.[87] Similar exacerbations and recurrences of individual psychotic patterns are seen following administration of the methyl donors, methionine[88] and betaine.[89] These findings, together with the known psychotomimetic effects of dimethyltryptamine[90] and bufotenin[91] led several authors[88,92] to suggest that abnormal methylation of indoleamines, leading to the endogenous formation of methylated indole(ethyl)amines, may be a primary causative factor in the pathogenesis of schizophrenia. This has been supported by the recent isolation of a specific indoleamine N-methyltransferase from rat and human brain,[93] and the demonstration that tryptamine is converted to dimethyltryptamine in rat and human brain.[94] This theory is also compatible with a recent α-2 globulin theory,[95] which postulates an enzyme abnormality that permits excess Trp to enter brain.

Clinical attempts to elevate CNS 5-HT levels in schizophrenics have generally had deleterious effects, particularly when L-Trp is combined with an MAO inhibitor.[87] Reports of mild beneficial effects of L-Trp[74] and L-5-HTP[96] in schizophrenics have appeared recently.

Other Behavioral States - Elucidation of the role of 5-HT in sleep[26b,27] has prompted numerous clinical studies with L-Trp and L-5-HTP. L-Trp appears to sedate normals and insomniac patients[97] and has recently been described as a "natural sedative".[98] Clinical studies of L-5-HTP in sleep have not been as impressive.[99]

Several behavioral disturbances including psychoses frequently accompany Hartnup's disease, Wilson's disease, porphyria and pellagra, diseases in which indoleamine abnormalities have been identified.[100] These behavioral aberrations generally recede during clinical remission of the primary metabolic abnormality.

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Section II - Pharmacodynamic Agents

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Chapter 6. Antihypertensive Agents

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Introduction - No major advance in our understanding of the nature of hypertension has been made this year, and it remains true that "until the mysteries of the etiology of essential hypertension are unravelled, treatment must remain empiric and palliative".[1] Several reviews[2-4] have discussed various aspects of the clinical conditions which are associated with an elevated blood pressure, and which are the target for so much research. Other reviews[5-9] have described the clinical benefits achieved by lowering elevated blood pressure; it is possible that this benefit is obtained by the reversal of the increased ratio of the vascular wall thickness to the diameter of the lumen which is a characteristic of essential hypertension.[10]

The spontaneously hypertensive rat (SHR) continues to gain attention as a model with which to assess antihypertensives.[10-12] The extent to which different anesthetics reduce cardiovascular responses of cats has been studied,[13] and the danger of assuming that drug solvents such as polyethylene glycol have no effect on these responses has been pointed out.[14]

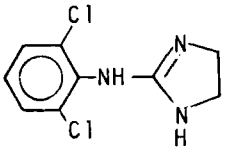
Developments in clinically useful antihypertensives - There has been a great deal of discussion[15] on work carried out to evaluate the therapeutic potential of combining a vasodilator with a β-adrenoreceptor blocker in order to counteract the tachycardia associated with the former. The combination of hydralazine with propranolol (p.o.) is effective in hypertensive patients at doses which separately do not give a satisfactory hypotensive effect;[16] a similar situation holds for dihydralazine.[17] Alprenolol (i.v.) also reduced the cardiac stimulation observed in normotensive and hypertensive patients after dihydralazine (i.v.).[18] Minoxidil (PDP), in combination with propranolol and hydrochlorothiazide, appears to be more effective in refractory hypertensive patients than hydralazine.[19] Practolol effectively blocks the tachycardia in normotensive dogs treated with hydralazine (i.v.) without potentiating the hypotensive effects, which suggests that tachycardia does not diminish the hypotensive effect of hydralazine.[20]

Guancydine has been examined in further clinical trials,[21,22] and shown to have no effect on the pressor response of angiotensin in hypertensives.[22] The mode of action of guancydine has been studied in vitro leading to the conclusion that its hypotensive properties result

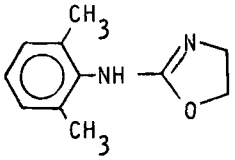
from its non-specific inhibitory effect on smooth muscle.[23]

The cardiovascular responses of patients receiving propranolol have been compared to those receiving bethanidine, guanethidine and α-methyldopa;[24] no advantage is gained by combining propranolol with these sympathetic inhibitors.[25] Propranolol is thought to act as an antihypertensive by decreasing the cardiac output and conditioning the baroreceptors to function at a lower level;[24] in addition to cardiac β-blockade, there may be a component of adrenergic neurone blockade in this action.[26] In contrast, the antihypertensive action of practolol may be due to vasodilation.[27] The problems involved in the clinical evaluation of these drugs have been discussed.[28]

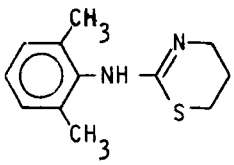
There has been a succession of reports this year on the clinical effects of clonidine (I), which can be used with advantage in conjunction with chlorthalidone.[29,30] The unique and complex mechanism of action of this drug is now becoming clear.[31] When all sympathetic transmission is blocked, a peripheral vasodilating activity has been uncovered,[32] in addition to its peripheral α-receptor stimulant action which results in a brief hypertensive response after i.v. administration at low doses. The action which predominates and makes clonidine such a useful drug is its ability to stimulate central α-receptors leading to an inhibition of sympathetic tone.[33,34] An effect on the central control of vagal activity, at least in dogs, has also been suggested.[35]



I



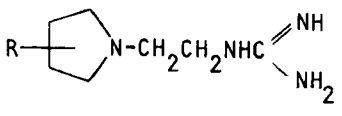
II



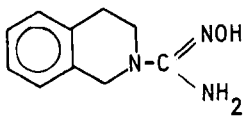
III

Studies on the hemodynamics[36] and central sites of action[37] of several α-receptor stimulants, including clonidine (I), the oxazoline (II, LD2855), and xylazine (III, BAY 1470) have been reported.

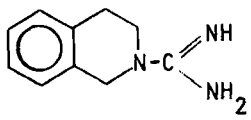
Adrenergic neurone blockers - Several methylpyrrolidine analogs (IV, R = $(CH_3)_2$ or $(CH_3)_3$) of guanethidine have activities comparable to that of guanethidine when tested p.o. and s.c. in renal hypertensive rats. (IV, R = cis-2,4-$(CH_3)_2$) is less active than guanethidine as a sympathomimetic in dogs and cats (i.v.).[38]



IV



V

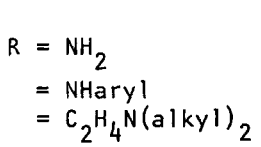


VI

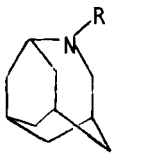
The N-carbamidoxime (V) lowers the blood pressure of renal hypertensive dogs at 2 and 8 mg/kg and is also reported to be an effective antihypertensive in neurogenic dogs, as well as renal and spontaneously hypertensive rats. This compound resembles guanethidine and debrisoquine (VI) in its adrenergic neurone blocking and catecholamine-depleting activity, but it may have an additional central effect.[11,39] Debrisoquine has now been shown to deplete that small part of the norepinephrine store which has been postulated as essential for the proper functioning of adrenergic neurones.[40]

A comparison of the antihypertensive effect of guanethidine with three of its metabolites in renal hypertensive rats leads to the conclusion that guanethidine *per se*, and not its metabolites, is responsible for its hypotensive effects.[41]

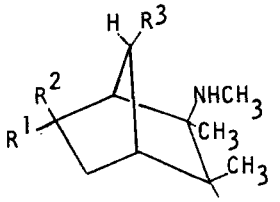
Ganglion blockers - At 10 mg/kg, derivatives of 4-azahomoadamantane (VII) and (VIII) lower the blood pressure of anesthetized dogs by at least 15% for two hours or more, apparently by ganglion blockade.[42] The monomethoxymecamylamines (IX, R^1, R^2, or R^3 = CH_3O) are about as active as mecamylamine in lowering the blood pressure of renal hypertensive rats, the corresponding hydroxy derivatives are less active.[43]



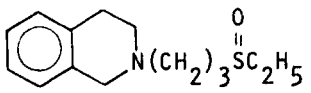
VII



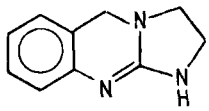
VIII



IX



X

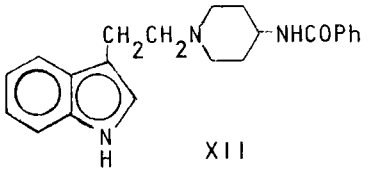


XI

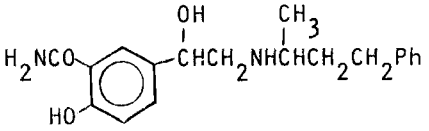
α-Adrenoreceptor blockers - Further details have now appeared[44] on the cardiovascular effects of the α-blocker (X, NC 7197) mentioned last year.[45] The tricyclic guanidine (XI), which significantly lowers the blood pressure of metacorticoid rats at 5 mg/kg, and conscious normotensive or neurogenic hypertensive dogs at 2.5 mg/kg, probably has α-blockade as its main mechanism of action. However, since there is no orthostatic hypotension in the rabbit tilt test, its mechanism of action must be complex.[46]

Indoramine (XII, Wy 21901) abolishes epinephrine- and ouabain-induced cardiac arrythmias by a combination of α-adrenoreceptor blockade and membrane stabilization.[47] These effects have also been studied electrophysiologically.[48] Indoramine has a pA_2 of 7.4 as a competitive α-blocker on aortic strip, and a pA_2 of 8.2 as an antihistamine on guinea pig ileum.[49] The blood pressure of cats and conscious renal hypertensive rats is lowered by indoramine at 20-40 mg/kg (p.o.); several analogs are also active but rather toxic.[50] A dose-related reduction in blood pressure accompanied by an increase in heart rate after administration of indoramine has been observed in man.[51]

The new compound (XIII, AH 5158A) is particularly interesting because it combines significant β-adrenoreceptor blocking activity with α-blocking activity both in animals and in man.[52]

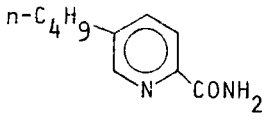


XII

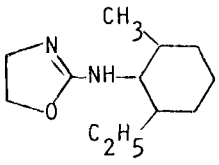


XIII

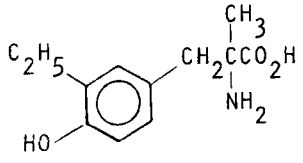
Compounds affecting adrenergic transmitters - Fusaric acid amide (XIV, Sch 10595) is a very active inhibitor of dopamine-β-hydroxylase *in vivo* (but not *in vitro*), and it lowers the blood pressure of DOCA hypertensive rats for many hours in a dose-related manner.[53] The cyclohexylamino-oxazoline (XV, BAY a6781) probably exerts its hypotensive effect by preventing the release of catecholamines from the adrenal gland.[54] 3-Ethyl-α-methyltyrosine (XVI) is a competitive inhibitor of tyrosine hydroxylase and exhibits significant antihypertensive activity in DOCA rats at 25 mg/kg. It may be preferable to α-methyltyrosine because it should not be metabolised into a catecholamine.[55]



XIV



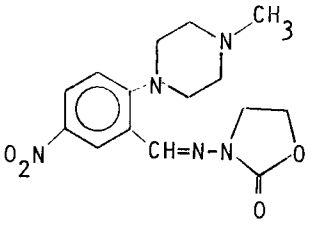
XV



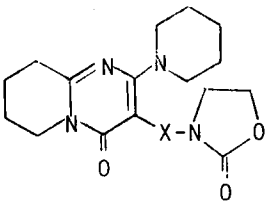
XVI

The mechanism of the central hypotensive effects of L-dopa, α-methyldopa and m-tyrosine has been studied with the aid of peripheral decarboxylase inhibitors.[56,57] The effects of L-dopa on the cardiovascular system have been reviewed.[58] A comparative study of the dose-dependent antihypertensive actions, and the norepinephrine-depleting activities, of α-methyltyramine and α-methyloctopamine in renal rats has been published.[59]

A series of hydrazones has been shown to have antihypertensive and monoamine oxidase inhibitory activity, although there was no correlation between these actions. The most active compound (XVII) caused a prolonged fall of 40-60 mm Hg in the blood pressure of anesthetized dogs and cats at 1 mg/kg (i.v.), and also in Goldblatt hypertensive rats at 30 mg/kg (p.o.) when given for 10 days.[60] In another study, the compounds (XVIIIa and XVIIIb) were the most active of a series of antihypertensive monoamine oxidase inhibitors.[61]



XVII

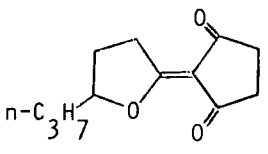


XVIII

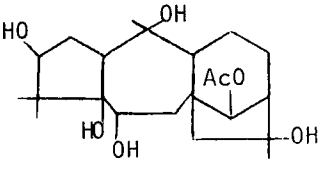
a) X = -CH=N-

b) X = $-CH_2NH-$

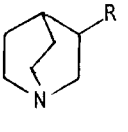
Naturally occurring and related compounds - The structure and synthesis of oudenone (XIX), the hypotensive mentioned last year,[45] has now been reported.[62] Acetylandromedol (XX, grayanotoxin I), obtained from various species of Rhododendron, is hypotensive in cats at 0.04 mg/kg (i.v.); artefacts with related structures are also hypotensive in cats or rats.[63]



XIX

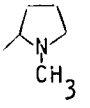


XX



XXI

a) R =

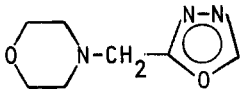


b) R =

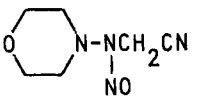
Analogs of nicotine and anabasine in which the pyridine ring has been replaced by quinuclidine (XXIa and both diastereoisomers of XXIb) show hypotensive effects when given i.v. to dogs.[64] The hydrazide of yohimboic acid, which is considerably less toxic than the methyl ester (yohimbine), causes prolonged hypotension at 1 mg/kg (i.v.) in chloralosed dogs.[65]

Prostaglandins - The effects of prostaglandins on cardiovascular responses would appear to be critically dependent on dose levels: infused into humans at the appropriate dose, PGA_1 and PGE_1 cause a fall in blood pressure and renal dilatation without the release of renin.[66,67] However, these effects are not simply dose related.[68] Experiments on the cat spleen have led to the suggestion that there is a continuous basal secretion of vasodilating prostaglandins;[69] at very low infusion rates of PGA_1 and PGE_1 in the dog, effects due to direct vasodilation can be separated from sympathetic inhibition.[70]

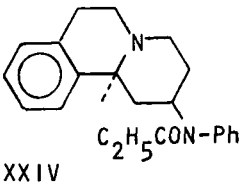
Compounds acting directly on vascular smooth muscle or by unknown mechanisms - The 1,3,4-oxadiazole (XXII)[71] and the acetonitrile (XXIII)[72] cause brief vasodilation in cats and in dogs respectively.



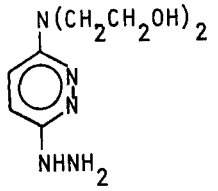
XXII



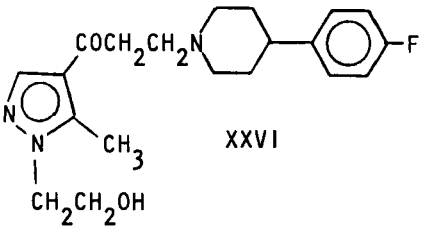
XXIII



XXIV

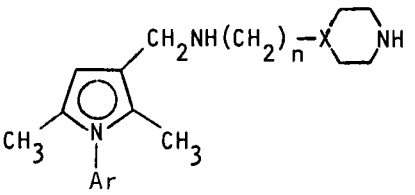


XXV

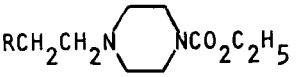


XXVI

The benzoquinolizine (XXIV) is a hypotensive vasodilator in anesthetized dogs and cats; it lowers the blood pressure of conscious renal hypertensive dogs at 10 mg/kg/day (p.o.).[73,74] The hypotensive effect of the hydrazine (XXV, L6150) in conscious or anesthetized dogs (0.5 or 0.01 mg/kg, i.v.) appears to be due to peripheral vasodilation.[75] The pyrazole (XXVI) is a potent vasodilator which reduces the blood pressure of cats and dogs by 40-50 mm Hg for 2 hours at 0.25 mg/kg (i.v.). It is also active in renal hypertensive rats.[76]

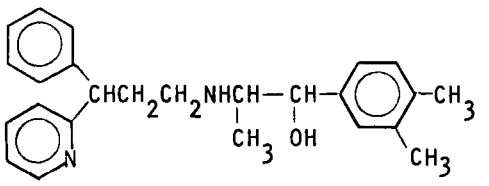


XXVII	Ar	n	X
a)	C_6H_5	1	CH
b)	C_6H_5	2	CH
c)	C_6H_5	2	N
d)	2,6-$Me_2C_6H_3$	2	CH
e)	C_6H_{11}	1	CH



XXVIII

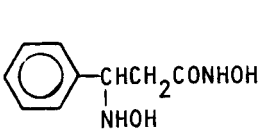
R = N-1,2,3,4-Tetrahydroisoquinolino
4-Benzyl-1-piperazino
4-m-Chlorophenyl-1-piperazino



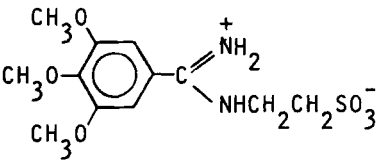
XXIX

A series of twenty-nine 3-pyrrolemethylamines (XXVII) show hypotensive activity in conscious and anesthetized dogs; (XXVII, a - e) reduce the blood pressure of the latter by 20 mm Hg at 1 mg/kg (i.v.) or less.[77] Other compounds reported to be hypotensive in anesthetized

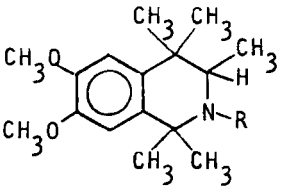
cats or dogs are the piperazines (XXVIII),[78] the prenylamine analog (XXIX, HOE 674),[79] the hydroxamic acid (XXX)[80] and the amidinium salt (XXXI).[81]



XXX



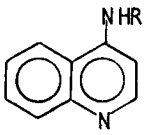
XXXI



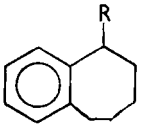
XXXII

The highly substituted tetrahydroisoquinolines (XXXII, R = $PhCH_2$ and CH_3) lower the blood pressure of anesthetized dogs by 30 mm Hg for 1 hour at 10 and 4 mg/kg (i.v.) respectively;[82] a reduction of 40% or more for at least 4.5 hours occurs after giving several 4-substituted aminoquinolines (XXXIII).[83] The benzocycloheptenes (XXXIV, a and b) show hypotensive activity in rats at 10 and 20 mg/kg (i.v.) respectively.[84]

XXXIII

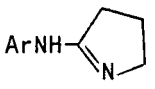


R = e.g. H, alkyl, furfuryl

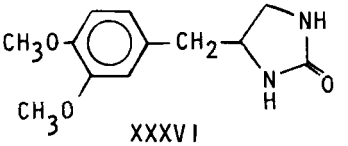


XXXIV

a) R = 4-(1-methylpiperazinyl)
b) R = $N(Ph)CH_2CH_2N(CH_3)_2$



XXXV



XXXVI

The amidine analog (XXXV, Ar = $2,6\text{-}Cl_2C_6H_3$) of clonidine, and several related compounds (e.g. XXXV, Ar = C_6H_5, $2\text{-}CH_3C_6H_4$, $3\text{-}CH_3C_6H_4$, $2,3\text{-}(CH_3)_2C_6H_3$, $2,4\text{-}(CH_3)_2C_6H_3$, and $2,6\text{-}Cl_2C_6H_3CH_2$) are active antihypertensives in DOCA rats at 10 mg/kg intragastrically.[85]

A detailed study has been made of the unique combination of properties exhibited by the hypotensive imidazolidine (XXXVI, Ro 7-2956). In dogs, a direct peripheral vasodilation is combined with a non-adrenergic myocardial stimulant activity reminiscent of theophylline.[86]

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Chapter 7. Antianginal Agents

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Angina pectoris is a painful symptom of myocardial ischemia occurring when the oxygen supply cannot meet the requirements of the heart. During ischemia, pain and abnormalities of heart metabolism, left ventricular function and electrocardiograms are observed.[1] Blood flow through the myocardium is the main determinant of oxygen supply.[2] The heart extracts oxygen from its blood supply at near maximal efficiency so that any increase in oxygen requirements must be met by an increase in blood flow. Ischemia caused by an insufficient oxygen supply may be improved by agents such as nitrates which increase myocardial perfusion and reduce oxygen utilization.[3]

Early work with nitroglycerin indicated a coronary vasodilator action which led to a concerted effort to find more potent, longer acting vasodilators selective for the coronary vascular bed. These newer agents have been disappointing since little relief of ischemia or anginal pain has been observed.

Another more recent approach to treatment of angina[4] includes the reduction of myocardial oxygen requirements through reduced cardiac work. Also, improved circulation of blood to ischemic areas by selective dilation of certain larger supply vessels appears to restore normal oxygen and nutrient supply. Circulation through the left ventricle involves epicardial (superficial regions) and endocardial (deeper regions) pathways which compete for available blood flow from larger intramural supply vessels. Further branching of the supply vessels leads to prearteriolar, arteriolar and capillary beds which have a nutritive function in the tissues. It has been suggested that circulation in the endocardium is probably inadequate, thus establishing a relatively ischemic region.[3,5] Compared with the epicardium, the endocardial region has a lower oxygen tension and a lower level of circulating nutrients. When coronary artery disease restricts blood flow, the endocardial region may be prone to myocardial insufficiency. Since the endocardium is relatively ischemic, autoregulatory responses cause an average arteriolar and precapillary sphincter dilation of about 91%. The vessels of the better perfused epicardium are only opened about 68% and can better tolerate a decreased flow through compensatory mechanisms.

Nitroglycerin - Nitroglycerin is the oldest and most effective antianginal agent in use today. Used prophylactically or for immediate relief of anginal pain, nitroglycerin acts by reducing heart work and by redistributing blood flow to the ischemic areas of the myocardium. The beneficial effect of nitroglycerin on the myocardial circulation of dogs is the result of a number of actions.[6,7,8,9] The reduced ischemia is probably the result of a decreased oxygen requirement due to a reduced heart work, filling pressure and stroke volume. The major mechanism of action appears

to be an increased oxygen tension in the endocardium due to a redistribution of blood from the epicardium.[4,5,10,11] While myocardial blood flow is decreased by nitroglycerin, endocardial perfusion is maintained at pretreatment levels by the redistribution of blood from the epicardium. Endocardial oxygen tension rises as a result of the redistributed blood flow and decreased oxygen requirement. Nitroglycerin redistributes blood flow to the endocardium by dilating the larger supply or intramural vessels controlling the flow distribution between the inner and outer regions of the myocardium. The intramural vessels do not appear to be controlled by the autoregulatory mechanism so that they respond differently to drugs than arterioles. Measurements of changes in large vessel resistance and oxygen tension correlate well with the effects caused by nitroglycerin. Microsphere distribution studies in the dog also indicated a redistribution of flow to the endocardium due to nitroglycerin action.[10]

In contrast to nitroglycerin and pentaerythritol tetranitrate, coronary vasodilators such as dipyridamole[4] and chromonar[12] increased total coronary blood flow in dogs without redistributing blood flow or improving endocardial oxygen tension. The coronary vasodilators, dipyridamole, chromonar, lidoflazine, iproveratril, papaverine and prenylamine were all shown to exert a selective dilator action on small coronary arteries and arterioles.[13]

It has been suggested[14] that the smooth muscle relaxant action of nitroglycerin may be related to its initial blood concentrations rather than a certain optimum blood level. Nitroglycerin appears to be most effective in abolishing existing contractions of vascular smooth muscles rather than preventing their contraction. This may indicate why nitroglycerin is therapeutically more effective sublingually or by subcutaneous injection than orally.

Blum[15] was unable to demonstrate a relationship between reduced oxygen utilization, inhibition of mitochondrial respiration and dilatation of large coronary arteries using a series of organic nitrates. Biochemical evaluation did not consistently predict which agents would affect blood flow to the endomyocardium.

Coronary Vasodilators - Continued interest in this approach to antianginal agents has been reflected by the numerous reports of agents increasing coronary blood flow and oxygen supply. These agents probably act by interfering with metabolic autoregulation of coronary blood flow.[16,17] Autoregulation is a mechanism by which the heart maintains sufficient blood flow and nutrient supply for its metabolic activity. Some vasodilator agents appear to be specific dilators of smaller coronary blood vessels by an adenosine-sparing mechanism. The end product of adenosine triphosphate (ATP) breakdown in the heart appears to be adenosine while in other tissues such as skeletal muscle, inosine is the product.

Adenosine, a potent vasodilator, has been shown to be present in the dog heart in nucleosidic and nucleotidic forms. During ischemic conditions, both myocardial and blood levels of adenosine are increased.

Although not proven conclusively, adenosine has been implicated as at least one mediator of autoregulatory control of myocardial blood flow. Oxygen tension, potassium ion concentration, blood pH and a myogenic response also may be involved in autoregulation. However, a change in adenosine concentration as a result of ischemia can fully account for vasodilator responses through autoregulatory control.[17]

Any thing which may help create an oxygen deficiency such as active transport processes, increased heart work or impaired coronary circulation due to occlusion or cardiovascular disease also decreases the ATP supply in the myocardium. During ischemia, the equilibrium between ATP and adenosine diphosphate (ADP) favors ADP formation and adenosine. The higher intracellular adenosine concentration promotes diffusion of adenosine through cellular membranes into the blood of the myocardium. A resulting localized vasodilation increases the blood flow and oxygen supply to the ischemic area. The increased ATP/ADP ratio permits more work and metabolic processes to be supported. Blood flow is maintained at a given level of metabolic activity and adenosine concentration.

Coronary vasodilators interact with the adenosine-mediated vasodilation response by preventing the deamination or reabsorption of adenosine present in the blood. Such agents are dipyridamole,[18,19] hexobendine,[20] lidoflazine,[18] 2-alkylthioadenosines,[21] and other substituted-adenosines[22] in dogs and pteridine[23] analogs in kittens. Although these agents are inhibitors of intracellular adenosine deaminase, it is believed that their primary action is the inhibition of adenosine diffusion across cellular membranes where it is deaminated.[16]

Adenosine uptake may involve a membrane-bound kinase enzyme which releases adenosine as its monophosphate within the cell.[24] Dipyridamole inhibits this membranal kinase in guinea pigs to a greater degree than soluble intracellular adenosine deaminase or kinase enzymes. Dipyridamole, in rats, does not prevent uptake or potentiate the vasodilator effects of adenosine due to a lack of the membranal kinase transport mechanism. Thus, species variation in adenosine uptake mechanism may tend to confuse studies in mechanisms of action with vasodilators interfering with adenosine.

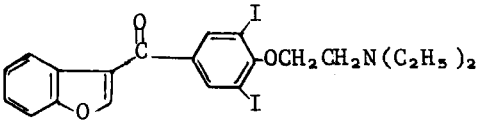
Ischemia may cause deterioration of myocardial function through excessive loss of ATP and the myocardial pool of purine nucleosides. The enzyme, xanthine oxidase, irreversibly converts purines to uric acid. After periods of asphyxia, an infusion of adenosine caused a markedly accelerated restoration of normal myocardial nucleotides.[25] In coronary-ligated dogs and sheep, the xanthine oxidase inhibitor allopurinol prevented or reversed nucleotide loss from the myocardium, ST-depression and cardiac arrhythmias due to myocardial ischemia.[26]

Hexobendine[27] causes a metabolic acidosis which may contribute to its vasodilator action in humans.

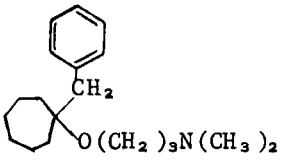
Other agents such as amiodarone,[28] bencyclan,[29] chromonar,[30]

diazoxide,[31] iproveratril,[30] perhexiline,[32] and diazepam[33] have been shown in dogs to exert a vasodilatory action on small coronary blood vessels. The mechanisms of action have not been further elucidated. These agents also possess a peripheral vasodilator action which may be beneficial in anginal patients by decreasing blood pressure, venous return and heart work.

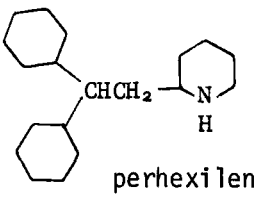
An understanding of the microcirculatory responses of the heart to nitroglycerin and coronary vasodilators clearly indicates why nitroglycerin is an effective antianginal agent, while vasodilators are generally ineffective or deleterious in ischemic conditions.[4,5] Nitroglycerin dilates larger coronary arteries not controlled by autoregulation mechanisms. A redistribution of blood flow results which diverts blood from the normal epicardium to the ischemic endocardium. Little change in total coronary flow is observed. Since coronary vasodilators act by interference of autoregulation mechanisms in smaller coronary vessels, vasodilation of the normal epicardial vessels probably predominates. The smaller vessels in the ischemic endocardium are probably maximally dilated due to anoxia. Thus, an enhanced epicardial flow must divert blood flow from ischemic areas since they compete for available flow. Coronary vasodilators could not be expected to be beneficial but may be harmful when used to treat myocardial ischemia and angina pectoris. In very severe ischemia, high doses of dipyridamole actually induced anginal attacks in man.[34]



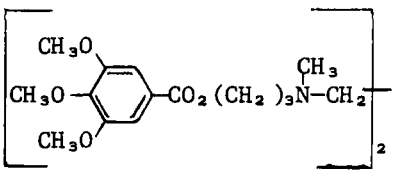
amiodarone



bencyclan



perhexilene



hexobendine

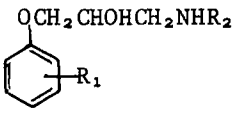
Beta-Adrenergic Blocking Agents - Treatment of angina pectoris with β-blocking agents is a more recent approach which appears to be of therapeutic value. Exercise and stress tend to increase catecholamine levels and cardiac sympathetic drive in anginal patients. Excessive sympathetic stimulation creates an oxygen wasting effect by increasing oxygen use in the myocardium more than is required to perform additional work. β-Blockade prevents this excessive oxygen consumption and reduces cardiac work.[35] The lower cardiac work and oxygen requirement allows the heart to perform more efficiently during moderate exercise at a lower heart rate.

A number of β-adrenergic blockers have recently been reported in the literature. Two previous reviews have covered all but the most current analogs.[36,37] Ring substitution ortho to the oxypropanolamine side chain by cyclopropyl (SD-2124-01) has led to a nonselective β-blocker of potency similar to prindolol and greater than propranolol in dogs. This analog lacks significant β-sympathomimetic and cardiodepressant activity but is capable of reversing ouabain-induced cardiac arrhythmias possibly by facilitating atrio-ventricular conduction.[38]

Two nitrile-substituted analogs, Ko-1313 and Ko-1366, have been reported[39,40] as nonselective β-blocking agents which were more potent than propranolol in dogs and also reversed ouabain-induced cardiac arrhythmias. However, significant depression of atrio-ventricular conduction was observed at antiarrhythmic dosage levels.

Another analog similar in potency to propranolol is D-69-12. This agent is active in the guinea pig.[41] The compound D563 or l-3-methoxy-ω-(l-hydroxy-l-phenylisopropylamino)-propiophenone is a β-blocker in dogs.[42]

In addition to practolol, other p-acylamino analogs[43] were shown to be cardioselective β-blockers and more potent than the parent compound. Para-allyl and -allyloxy substituted analogs of alprenolol and oxprenolol have been reported to be cardioselective in cats.[44]



	R_1	R_2
SD-2124-01	2-cyclopropyl	t-butyl
Ko-1313	2-CN	isopropyl
Ko-1366	2-CN	t-butyl
D-69-12	2-Cl	t-butyl
practolol	4-$NHCOCH_3$	isopropyl
alprenolol	2-$CH_2CH{=}CH_2$	isopropyl
oxprenolol	2-$OCH_2CH{=}CH_2$	isopropyl
KL-255	2-Cl, 5-CH_3	t-butyl

β-Adrenergic blockers remain contraindicated in those patients suffering from some myocardial decompensation or heart failure.[45] In those cases, β-blockade of normal sympathetic support of heart function may precipitate cardiac failure. Several studies concerning the cause of myocardial depression and induction of heart failure have been reported.[46-49] β-Blockers which possessed significant cardiac depressant properties may have interfered with calcium transport across membranes of the mitochondria and sarcoplasmic reticulum in dog heart muscle. This resulted in a depression of the contractile state of the myocardium.

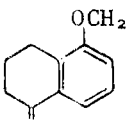
Nayler[46] proposed that agents which have significant β-sympathomimetic action did not interfere with calcium transport and the contractile state. They were less depressant on the myocardium than β-blockers not having β-stimulant action. The depressant effect was not related to β-blocking potency. At high concentrations, Noack and Greeff[47] demonstrated that β-blockade was not involved in inhibition of calcium transport. Using an *in vitro* model, *d*- and *l*-alprenolol were equally effective inhibitors of calcium transport.

After a study of the cardiac depressant action of KL-255, it was suggested that myocardial depression may be a result of β-blockade of the basal tone established by endogenously released catecholamines.[48] Catecholamine depletion by guanethidine resulted in a loss of the depression of contractile force normally observed with KL-255. The depressant effects did not appear to be dose-related to local anesthetic action.

Intrinsic β-sympathomimetic activity of some β-blockers did not appear to prevent the onset of cardiac failure in hearts dependant upon sympathetic drive. It was suggested that β-blockers with significant β-sympathomimetic action, such as DCI, might be less likely to precipatate heart failure. The more depressant propranolol lacks β-sympathomimetic activity. No difference was observed between DCI and propranolol when introduced during the sympathomimetic-dependant phase of experimental heart failure in guinea pigs.[49]

Although β-blockers tend to reduce coronary blood flow, they may not be harmful to patients with myocardial ischemia. In the dog, propranolol reduces blood flow to normal but not to ischemic areas of the heart. Using Rb^{86} uptake, Xe^{133} clearance and labelled microsphere distribution studies, experiments show that propranolol, like nitroglycerin, redistributes blood flow into ischemic regions of the heart.[10,50-52]

Winbury[53] used oxygen tension to reflect increased regional perfusion and decreased metabolic requirement of oxygen. The β-blockers, bunolol and propranolol, increased oxygen tension in ischemic areas of the dog endocardium. Redistribution of blood flow into deeper areas of the endocardium appears to be a common action of both nitroglycerin and at least some β-blockers.



bunolol

Clinical Therapy - The variables in clinical evaluation of antianginal agents are well documented. Unless the drug studied is 100% effective, judgement of therapeutic efficacy is likely to be mixed with bias or subject to varied interpretations among investigators. It is common to see agents effective in some studies but no better than placebo in others.

Variations in dosages studied, as well as duration of action, often lead to inconsistent results. Isosorbide dinitrate is an active antianginal agent with a duration of action similar to nitroglycerin. Some reports of ineffectiveness may have been due to the ineffective dosage

studied or the agent's short duration of action.[54-57] Chronic treatment with isosorbide dinitrate did not cause a cross tolerance with nitroglycerin to develop.[57]

Nitroglycerin continues to prove useful in angina treatment. Using reduced ST-depression as a criterion for agent effectiveness in ischemia, it was shown that nitroglycerin was beneficial while several coronary vasodilators including chromonar, dipyridamole and hexobendine were ineffective in long term therapy.[58,59]

β-Adrenergic blocking agents continue to show utility in the treatment of angina if administered to carefully selected patients.[35,58,60,61] Anginal pain induced by isoproterenol or exercise was prevented or eliminated upon treatment with propranolol or oxprenolol. Ischemic changes noted as ST-depressions were reduced by β-blocker therapy. Alprenolol, however, was reported ineffective in a separate study.[62]

In a trial with patients having myocardial infarction,[63] the cardioselective β-blocker practolol showed possible value in the prevention of arrhythmias associated with severe ischemia while it did not induce clinical deterioration or failure of the myocardium. Propranolol, however, did cause deterioration in some patients.

Combination therapy with β-blockers and nitrates has continued.[35,64] Combined therapeutic effects have reduced the positive inotropic and chronotropic effects of nitroglycerin and the vasoconstrictive action of β-blockers. Beneficial synergistic effects upon reduced cardiac work, oxygen requirements, and improved flow to ischemic areas of the myocardium have been observed. Combinations of alprenolol or propranolol and isosorbide dinitrate significantly increased work tolerance and delayed pain and ST-depression in some angina patients. The individual drugs were less effective. However, in another study, isosorbide dinitrate was ineffective alone or in combination with propranolol.[65] In the same study, propranolol induced heart failure in some patients where no clinical deterioration had been detected earlier.

A small synergism was noted when erythritol tetranitrate was combined with propranolol or alprenolol. It was suggested that further investigations involving larger numbers of patients are needed to evaluate the possible significance of combined therapy.[66]

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Chapter 8. Antithrombotic Agents

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A survey of research on platelet aggregation inhibitors[1] was presented in this series in 1971. A more comprehensive review of agents affecting thrombosis[2] appeared during the preceding year. The present report is intended as a continuation of these earlier works and attempts to summarize the significant recent research on all types of drugs for the management of thromboembolic disease. Due to space limitations, important related research on diagnostic techniques and experimental models of thrombosis will not be reviewed.

Approaches to Antithrombotic Therapy

As Schor[2] pointed out, there are three therapeutic approaches to the treatment of thrombosis: inhibition of platelet aggregation, inhibition of blood coagulation, and enhancement of the fibrinolytic process. The agents involved affect platelet function, or act on the blood coagulation mechanisms either by inhibiting the formation or enhancing the breakdown of fibrin. In this regard, it is important to differentiate between a clot and a thrombus.[3-5] A clot is a relatively disorganized, dark-red structure that results from the coagulation of whole blood and is usually associated with venous circulation. The structural integrity of the clot is maintained by a fibrin network and the principal event in clot formation is the activation of fibrinogen to fibrin. It contains red cells, white cells and platelets in roughly the same proportions as they occur in whole blood. Thrombi are simpler, more highly organized structures that occur with varying degrees of complexity,[4] but the usual form on the arterial side of the circulation is a white head composed almost entirely of aggregated platelets, to which is attached a red tail of fibrin containing red cells. The principal events in thrombus formation are initial adherence and the propagation of platelet aggregation by ADP resulting from the platelet release reaction.[6]

A variety of clinical states are associated with thrombosis. The present discussion is limited to a few of the more prominent thromboembolic diseases; further information on this subject may be found in several reviews.[3,7-9] The most common clinical states which involve clot-like, venous thrombi are deep vein thrombosis, particularly as a postsurgical complication, and pulmonary embolism. Anticoagulants such as heparin and the coumarins have been known for years to be effective in the prevention of these types of thrombi and more recent experience has demonstrated the efficacy of fibrinolytic agents such as streptokinase for their dissolution.[3,9] Platelet aggregation inhibitors have only recently been evaluated clinically in the prevention of venous thrombosis. These studies are crucial to the resolution of the controversy as to whether platelets play a vital role in the initiation of venous thrombi.[10] There is persistent histological evidence that indicates venous thrombi begin as platelet aggregates.[11] Myocardial infarction and stroke are

the major clinical manifestations of arterial thrombosis. Anticoagulants have been found to offer little, if any, benefit to the survivors of myocardial infarction, and prophylactic treatment with these agents is overshadowed by the danger of hemorrhage.[3] Large scale clinical studies of the fibrinolytic agent streptokinase in myocardial infarction have been undertaken in recent years.[12] Although a statistically significant improvement is indicated by these trials, the commitment to further studies to verify efficacy has been criticized on the basis of the limited possible benefits of fibrinolysis to the patient due to the critical time element involved in myocardial infarction. Since tissue necrosis distal to the coronary occlusion occurs within a few hours, it has been argued that little is to be gained by restoration of blood flow after this irreversible damage is done, and that clinical efforts would be better expended on prophylactic agents.[13] The results of streptokinase trials currently in progress should resolve the controversy and establish an important precedent in regard to the feasibility of fibrinolytic therapy in arterial thrombosis. In general, there appears to be agreement that platelet aggregation inhibitors are the most rational type of agent for the prophylaxis of arterial thrombosis. Aspirin, the pyrimido-pyrimidines related to dipyridamole (Persantine) and dextran are the most notable agents for which anti-platelet activity has been demonstrated *in vivo* in man. However, no major clinical trials of platelet aggregation inhibitors in pathologic states such as myocardial infarction have been reported. Moreover, selective platelet aggregation inhibitors that are lacking other major drug effects, such as antiinflammatory or hypotensive activity, have not undergone clinical evaluation. It is this latter class of agents that appears to hold the most promise for the prevention of arterial thrombosis.

Platelet Aggregation Inhibitors

Prostaglandins and Cyclic AMP - The effects of natural prostaglandins on platelet function have been studied extensively. As a group, they are generally inhibitors of ADP-induced platelet aggregation. PGE_1 is by far the most potent by at least two orders of magnitude. PGE_2 differs in that it exhibits a biphasic activity; at concentrations less than those required for inhibition, it potentiates platelet aggregation.[14] PGE_1 also inhibits clot retraction.[15] This potent effect may be attributed to inhibition of the platelet release reaction.[15] The possible clinical utility of PGE_1 has been suggested, although short duration of action appears to be an important limitation.[16] The lethal effect of intravenously administered ADP to rats, which appears to cause death by occlusion of the cerebral arteries with platelet thrombi, was prevented by PGE_1. Transient thrombocytopenia induced by ADP injection was also inhibited.[17] The growth of a particular type of platelet thrombus formed by electric stimulation in cortical veins was inhibited by locally administered PGE_1.[18] PGE_1 is stable and retains platelet activity on incubation with whole human blood.[14] Recently, a procedure has been developed for stabilizing platelet fractions with PGE_1 in blood banks. Platelets treated in this manner exhibited normal hemostatic function following transfusion in man.[19]

Two reports describe analogs more potent than PGE_1. The ω-homolog of PGE_1 has been shown to be about 4 times more potent than PGE_1 in the inhibition of platelet aggregation *in vitro*.[17] Similarly, an analog of PGE_1 with a *trans* double bond at the 2-position was found to be 2 1/2 times more potent than PGE_1. It is interesting that a number of isomers of this compound with the double bond at different positions in the side chains, including natural PGE_2, were all much less potent than PGE_1.[20]

Perhaps the most significant event in prostaglandin research during this past year was the discovery that aspirin inhibits the synthesis of prostaglandins in a variety of types of cells, and that this effect may account for many of the familiar actions of aspirin.[21] The prostaglandins PGE_2 and $PGF_{2\alpha}$ are products of the platelet release reaction induced by thrombin. Although aspirin inhibits the release reaction, relatively high concentrations of thrombin caused unimpaired release of platelet constituents in the presence of aspirin without the concomitant release of PGE_2 and $PGF_{2\alpha}$. This inhibition of prostaglandin production was also demonstrated with platelets from donors dosed with aspirin 1 hour before blood was taken.[22] Presumably, inhibition of prostaglandin synthesis does not contribute substantially to the inhibition of platelet aggregation by aspirin.

Evidence continues to support the hypothesis that platelet aggregation and the release reaction are inhibited by raised levels of intracellular cyclic AMP.[1] The inhibitory effects on platelet function of such diverse agents as PGE_1, dipyridamole, the methylxanthines and papaverine all appear to be mediated by this cyclic nucleotide.[23-28]

Aspirin - Several non-steroidal anti-inflammatory agents are known to inhibit platelet aggregation by preventing the release reaction. Therefore, aggregation induced by agents such as collagen, epinephrine and low concentrations of thrombin is inhibited, but ADP-induced aggregation is not affected. Among known inhibitors of this kind, which include salicylate, pyrazole and indoleacetic acid type anti-inflammatory agents, the most notable are indomethacin and aspirin.[1,3] Although reports on the platelet effects of indomethacin[29] and analogs such as "BLR-43"[30] continue to appear, the vast majority of the literature in this area is devoted to studies of aspirin.

Although aspirin and salicyclic acid exhibit half-lives in man of only 13 to 19 min. and 3.5 to 4.5 hrs., respectively,[31] the effect of aspirin on platelets *in vivo* has a duration which approximately parallels platelet survival time of 2-7 days. The currently accepted hypothesis to explain this action holds that aspirin irreversibly alters platelet function by acetylation of a protein component of platelet membrane. A substantial amount of experimental evidence consistent with this hypothesis has accumulated.[1,32] Although aspirin does not inhibit ADP-induced aggregation its effect on the release reaction decreases platelet response and abolishes the "second wave" of aggregation in the presence of ADP. It appears that another component of aspirin action is an effect on a plasma cofactor of ADP-induced aggregation.[33,34] Evidence has been

presented that Hageman factor is necessary for platelet-aggregate stability. In addition, the aggregating platelet surface has been proposed as a site of Hageman factor activation. Aspirin may inhibit these interactions by blocking the appropriate site on the platelet surface.[35,36]

The antithrombotic effect of aspirin has been demonstrated in a number of experimental animal models. The death rate due to pulmonary thromboembolism induced in mice by infusion of a collagen suspension was decreased reproducibly by aspirin.[37] Aspirin provided significant protection against the vasoconstrictive effects and mortality of pulmonary embolism caused by injection of fresh, autologous clots into rabbits[38] or dogs.[39] Presumably, suppression of the venoconstrictive component of pulmonary embolism by aspirin was due to the inhibition of serotonin release from platelets.[38] Occlusive thrombus formation induced in isolated arteries of dogs by mechanical or chemical injury was substantially reduced by aspirin ingestion. In this model dipyridamole had no effect.[40] Myocardial necrosis induced in dogs by infusion of epinephrine is believed to be caused by the formation of platelet thrombi. Both aspirin and dipyridamole have a significant protective effect against this phenomenon.[41] A new microiontophoretic technique has been developed for quantitating platelet aggregation *in vivo*. This procedure, was used to demonstrate that aspirin was several times more effective in the inhibition of hamster platelet aggregation *in vivo* than *in vitro*.[42]

A number of clinical trials with aspirin as an antithrombotic agent were reported during the past year. Some of the studies have involved venous thrombotic states in which the importance of platelet aggregation is uncertain at present. The outcome of the studies reported thus far does not furnish an adequate answer to this important question. In efforts to determine effective dose levels for the prevention of postoperative deep vein thrombosis, no antithrombotic effect was observed following the ingestion of as high as 2.4 g of aspirin daily.[43] A comparative study of aspirin, dipyridamole, dextran and warfarin was carried out in 169 patients undergoing hip reconstructive surgery. The high rate of occurrence of deep vein thrombosis which accompanies this type of surgery was significantly reduced by aspirin, dextran and warfarin, but not by dipyridamole.[44] A controlled study of morbidity and mortality among 430 aged people indicated that aspirin had no effect on thromboembolic disease in this particular group.[45] In hemodialysed patients aspirin was found to prevent fistula clotting which is characterized by increased platelet adhesiveness and thrombin production.[46] Platelet consumption associated with thromboembolic complications of prosthetic heart valves was found to be uniformly prevented by dipyridamole, but aspirin had little or no effect.[47] Some further examples of clinical states in which aspirin was found to have significant antithrombotic effect are spontaneous platelet aggregation as a cause of idiopathic thrombosis and recurrent painful toes and fingers,[48] the retinal-artery embolism of amaurosis fugax,[49] and thrombotic thrombocytopenic purpura.[50]

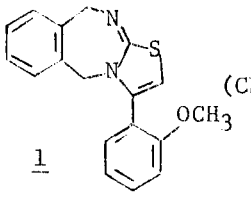
The present clinical evidence for the antithrombotic effect of aspirin is equivocal. Much further study will be required to demonstrate

efficacy, and once established, any antithrombotic benefit will have to be sufficient to outweigh the risk of gastric bleeding[51,52] on chronic administration to patients with thromboembolic disease.

Pyrimidopyrimidines - Dipyridamole and two analogs, RA233 and RA433, have been studied extensively as platelet aggregation inhibitors *in vitro* and in various animal models of thrombosis.[53,54] The mechanism of action of these compounds on platelets appears to involve inhibitory effects on glucose metabolism[53-55] and cyclic AMP phosphodiesterase.[27]

Clinical trials with dipyridamole for the reduction of thromboembolic complications after prosthetic cardiac valve replacement continue to show encouraging results.[47] In a controlled study of 163 patients, dipyridamole (400 mg daily) significantly reduced the frequency of arterial emboli.[56] On the other hand, dipyridamole was found to have no significant effect on postoperative deep vein thrombosis.[44]

New Agents - Reports of new structural types of platelet aggregation inhibitors have increased during the past year. The 5,10-dihydro-3-phenylthiazolo[3,2-b][2,4]benzodiazepine 1 and five phenyl-substituted analogs inhibited ADP-induced human platelet aggregation *in vitro* at concentrations near 10^{-5}M. Intravenous administration of 1 to rabbits resulted in inhibition of ADP-induced aggregation in subsequently drawn blood samples, and an oral dose of 1 in mice caused a significant increase in bleeding time.[57] Compound 2 was the most promising member of a large group of analogous acridine derivatives that inhibited ADP-induced platelet aggregation. This agent was active on human platelets *in vitro* at concentrations of 10^{-5}M. *In vivo* activity was demonstrated in rabbits.[58] Extensive synthetic manipulation of the ring substituents and the acridine nucleus gave a number of active analogs, but none was found to be superior to 2.[59] The antithrombotic activity of a new benzo[c][1,6] naphthyridine 3 was described. This compound inhibited ADP-induced aggregation *in vitro* at concentrations of 10^{-5}M. The effect was not reversed, but was significantly potentiated by epinephrine. Rat carotid artery thrombus formation induced by insertion of synthetic tubing was also inhibited.[60]



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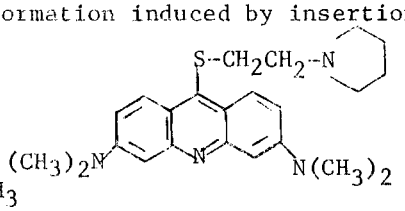

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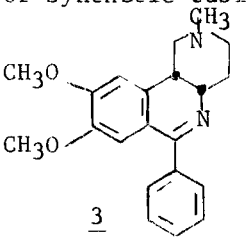
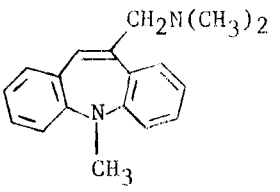


3

A comparative study of the inhibitory activity of 4 and 5 on biphasic ADP-induced aggregation of human platelets *in vitro* has been described. Compound 4 inhibited both phases of aggregation but was most effective on the second phase. In contrast, 5 inhibited only the second

phase which is associated with the release reaction. Compound 5 was about 400 times as effective as oxyphenbutazone.[61] Monodansylcadaverine (6), which is an effective inhibitor of the fibrin cross-linking enzyme fibrinoligase, was found to inhibit the second phase of ADP-induced platelet aggregation. Alkylation of the primary amine function resulted in analogs that retained platelet activity but fibrinoligase inhibitory activity was abolished.[62]



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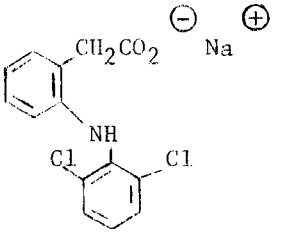

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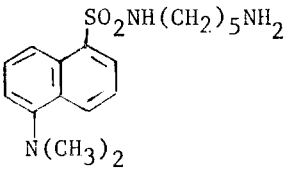
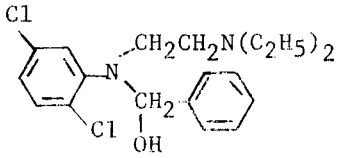


6

The aniline derivative 7 was found to be a potent inhibitor of ADP-, collagen- and thrombin-induced aggregation of human platelets *in vitro*. Oral administration of 7 to guinea pigs produced an inhibitory effect on platelet aggregability. *In vitro* concentrations of 7 comparable to *in vivo* platelet inhibitory concentrations had no effect on plasma coagulation factors, but higher concentrations were found to inhibit prothrombin and partial thromboplastin times.[63,64] A close analog of previously disclosed anticoagulants,[2] compound 8, has recently been reported as an inhibitor of ADP- and collagen-induced platelet aggregation and clot retraction. Platelet inhibitory concentrations of 8 did not affect blood coagulation.[65]



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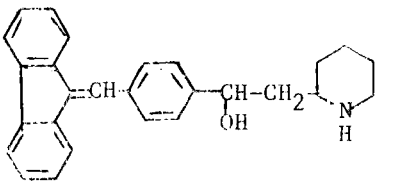
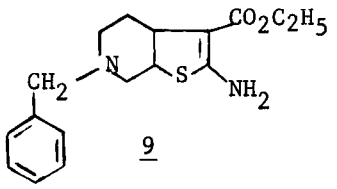


8

It has been shown recently that concentrations of collagen, insufficient to induce aggregation, potentiate ADP-induced aggregation of rabbit platelets. The novel tetrahydrothienopyridine derivative 9, which was described previously as an inhibitor of ADP-induced aggregation,[1] inhibited this potentiation. This observation was interpreted as evidence that 9 acts by inhibition of the release reaction.[66] Brief mention has been made of the fact that some derivatives (10) of the novel thiazolo-(3,2-a)pyrrolo(2,3-d) pyrimidine ring system are highly active inhibitors of ADP-induced platelet aggregation.[67]



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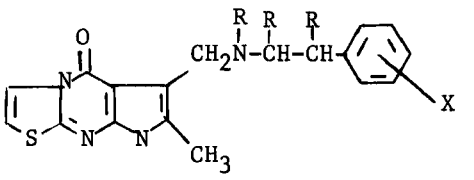

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Anticoagulants

The physiology and biochemistry of blood coagulation have been the subjects of several reviews during the past year.[68-70] Anticoagulants interrupt in some manner the complex mechanisms controlling the enzymatic conversion of a soluble blood protein fibrinogen into insoluble fibrin. Direct anticoagulants, such as heparin, inhibit the conversion of prothrombin to thrombin and the thrombin-fibrinogen reaction. Because of efficacy only by parenteral administration, heparin is used intravenously until oral anticoagulants begin to take effect.[9] The orally effective coumarin and indandione type anticoagulants act indirectly by inhibiting synthesis in the liver of blood clotting factors of the prothrombin complex. Vitamin K is essential for the synthesis of clotting factors in the liver, and a deficiency produces an anticoagulant effect similar to that of the coumarin drugs. The site of action of vitamin K and oral anticoagulants involves one of the final steps in the production of clotting factor subsequent to polypeptide chain synthesis on the ribosomal level. Inquiry into the nature of these steps and the precursors of the prothrombin complex is currently an active area of investigation.[71-74]

The comprehensive review by Douglas[9] provides a recent discussion of the clinically useful anticoagulants. Recent studies have shown heparin to be clearly effective in clinical states in which disseminated intravascular coagulation was indicated to be a pathologic factor.[75] The most commonly employed oral anticoagulants are of the coumarin type such as warfarin and nicoumalone. The oral anticoagulants appear to be of value in the prevention of thromboembolic complications after myocardial infarction.[9,76] However, anticoagulation therapy has been found to have no effect on death-rate in these patients.[77] Warfarin has been shown to be effective in the prevention of postoperative venous thrombosis.[44] The effectiveness of oral anticoagulants in the prevention of thromboembolism from prosthetic heart valves is still open to question. Recent experience with acenocoumarol and sodium warfarin in heart valve prostheses indicated a low incidence of thromboembolic complications.[78] Patients on oral anticoagulants are at considerable risk because of possible hemorrhage and the dangerous interactions between these agents and common drugs such as aspirin and the barbiturates. The complex subject of drug interactions with coumarin anticoagulants has been extensively investigated.[79,80]

Reports of new anticoagulants have been relatively rare. A new structural type found to have anticoagulant activity is the sodium salt of 2,3,5,6-tetrachloro-4-pyridinol. A number of analogs were found to be active, but less potent.[81] Some novel 4-hydroxycoumarin derivatives related to dicoumarol have been reported to have anticoagulant activity. The most potent was about half as active as dicoumarol.[82] Aromatic diamidines, which are known potent inhibitors of trypsin, inhibited human thrombin clotting activity and markedly increased prothrombin and partial thromboplastin times of human plasma. These compounds are also much more potent anti-fibrinolytic agents than ε-aminocaproic acid.[83]

Fibrinolytic Agents

A general discussion of fibrinolysis is available in recent reviews.[9,68,84] The oldest and most thoroughly studied fibrinolytic agent is streptokinase, a protein isolated from hemolytic streptococci. Similar to human activator, it directly activates plasminogen to plasmin. Although streptokinase is antigenic and frequently pyrogenic, these effects have not been sufficiently deleterious to preclude clinical utility.[3,9] Clinical experience with this agent has been gained now in most types of thromboembolic diseases,[85] with generally favorable results in the treatment of deep vein thrombosis (within 96 hours of onset of symptoms) and pulmonary embolism.[9,85,86] A major effort has been expended in recent years to investigate the usefulness of streptokinase in myocardial infarction, but results in this area are still open to question.[13,85,87-90]

Urokinase is a plasminogen activator prepared from human urine. It is produced by kidney cells and has been shown to differ from vascular plasminogen activator in terms of immunologic reaction, enzymatic activity, and molecular weight.[91] Although urokinase does not exhibit the antigenic side effects associated with streptokinase, development of this agent has been hindered by the expensive process required for extraction from urine. However, it appears to have superior therapeutic potential, and the claim has been made that cost could be reduced sharply if a large-scale demand were to develop.[90] A clinical trial to study the effects of urokinase on pulmonary embolism is currently in progress under the auspices of the National Heart and Lung Institute.[92] A recent study has demonstrated the feasibility of evaluating urokinase in myocardial infarction.[93]

Several other high molecular weight natural products have been investigated as fibrinolytic agents.[2] Ateroid is a natural mucopolysaccharide with fibrinolytic activity. The activation of fibrinolytic processes with this agent in man has been demonstrated in clinical studies. In addition, experiments in rats and rabbits have suggested that it may act by the inhibition of endogenous anti-activators. Recent evidence indicates one important component of the fibrinolytic action may be due to neutralization of antiplasmin.[94] A relatively non-specific proteolytic enzyme from *Aspergillus oryzae*, brinase, causes lysis of experimental thrombi faster than other clinical thrombolytic agents. Reduction of

serum antiplasmin activity by brinase may account for much of the in vivo fibrinolytic effect. Fibrinolytic effectiveness and a lack of untoward side effects has been demonstrated with brinase in a number of clinical states.[95] Arvin and reptilase are enzymes with similar action isolated from the venom of Malayan pit viper, Agkistrodon rhodostoma, and a South American snake, Bothrops atrox, respectively. These agents convert fibrinogen to an abnormal fibrin-like substance, which is more readily broken down to fibrinopeptides than fibrin and does not form intravascular clots. For the most part, clinical studies have demonstrated prophylactic effectiveness, but lysis of venous occlusions has also been claimed with arvin. Both arvin and reptilase inhibit the second phase of ADP-induced platelet aggregation.[3,96]

A compilation of synthetic drug approaches to fibrinolysis has been provided by Schor.[97] A variety of simple synthetic compounds are known to cause lysis of preformed clots. The majority of these compounds are antiinflammatory agents and structurally similar organic acids. They do not effect fibrin directly, but require the presence of serum proteins for clot lysis. Current evidence is consistent with a mechanism of action that involves inhibition of antiactivator or antiplasmin activity; but the possible direct activation of plasminogen has not been excluded.[97,98] This group of compounds also inhibits platelet aggregation with varying degrees of effectiveness. A comparative study of the fibrinolytic and platelet inhibitory effects has been carried out to try to optimize both activities in the design of one highly effective antithrombotic agent.[99] Bisobrin (EN-1661) is a bis tetrahydroisoquinoline derivative that has promising fibrinolytic activity in animals;[2,97] extensive structure-activity studies have been reported recently.[100] A peripheral vasodilator, bencyclane, has recently been reported to have potent fibrinolytic activity and to inhibit platelet aggregation.[101]

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Chapter 9. Pulmonary and Antiallergy Agents

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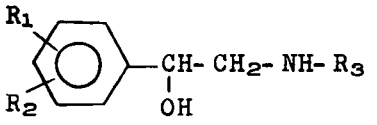
Introduction - In a reversible airway obstructive disease, such as asthma, respiration is impaired by bronchospasm, vasodilation, excessive mucous and edema. Various theories have been proposed to explain the etiology and pathogenesis of asthma. It has been categorized as an immediate type "hypersensitivity" resulting from an antigen-antibody (reagin) reaction with subsequent release of histamine, serotonin, bradykinin, slow-reacting substance (SRS-A) and possible other bronchoconstrictor substances. Since stress and brain lesions alter immunological reactivity and since emotional factors can exacerbate the clinical situation, the central nervous system and conditioning may play a role in asthma.. Vagal stimulation and cholinergic drugs can induce bronchial constriction by excessive parasympathomimetic tone and blockade by anticholinergic drugs has been useful in the treatment of asthma. Szentivanyi's[1] work has led to his theory of "blockade of the adrenergic β receptors" in the lungs as a possible cause of bronchial asthma. He postulates that the asthmatic has a defect at the β adrenergic receptor site which induces a state of hyperreactivity. This theory has been questioned because if asthmatics have an intrinsic β receptor blockade, they should find no relief with β adrenergic bronchodilators.

A new hypothesis about bronchial asthma has been introduced which is supplementary to the classical theories of the disease. It views the disease as the end result of interaction between decreased circulating epinephrine and a pathologically altered hyperactive bronchial tree[2]. Some clinical data[3,4] in bronchial asthma does indicate that there is a decrease in circulating epinephrine and this may be secondary to altered uptake or enzyme activity (COMT and MAO), or decreased release of epinephrine from the adrenal medulla. The decreased release of epinephrine from the adrenal medulla may involve the hypothalamus thereby linking the central nervous system with asthma. Drugs commonly used for symptomatic management act by a variety of mechanisms. Sympathomimetic agents (isoproterenol) mainly act by stimulating beta adrenergic receptors to cause bronchodilation and some (ephedrine and epinephrine) also stimulate alpha adrenergic receptors to cause vasoconstriction. The effectiveness of xanthine derivatives (aminophylline, theophylline) is based on their ability to relax the smooth muscle of the tracheobronchial tree and reduce edema of the bronchial mucosa. Their bronchodilator activity may involve the inhibition of lung cyclic AMP phosphodiesterase. Corticosteroids act through their ability to reduce inflammation and edema and alter tissue reactivity to

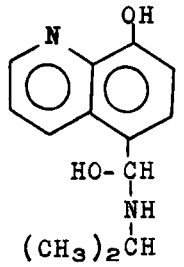
allergens. In an antigen-antibody reaction, histamine is released and while drugs that block histamine are successful in treating other allergic disorders, they have been ineffective in the treatment of asthma. This ineffectiveness could be related to the inherent ability of antihistamines to constrict smooth muscles[5]. Recently, the development of disodium cromoglycate has directed attention toward another potentially useful mechanism for the treatment of reversible airway obstructive disease. Disodium cromoglycate is not a bronchodilator, antihistamine or antiinflammatory agent. Instead, it is thought to act by blocking the release of allergy mediating substances such as histamine, and SRS-A resulting from antigen-antibody reaction. The prostaglandins, a series of naturally occurring hydroxy unsaturated fatty acids, have been reported to possess potent stimulatory or inhibitory properties on respiratory smooth muscle and indeed may offer a new class of drug to the existing group of bronchodilators.

Bronchodilators: Beta Adrenergic Stimulants - The diversity of actions of catecholamines at β adrenergic receptors led Lands[6] to postulate that there are two β receptor populations; β_1 mediating lipolysis and cardiac stimulation, and β_2 mediating bronchodilation and vasodilatation. Isoproterenol, a potent bronchodilator, activates β_2 adrenergic receptors but it also stimulates β_1 cardiac adrenergic receptors to cause tachycardia. Probably as a direct result of the cardiac stimulation, the hypoxaemia of the asthmatic patient could become worse when the drug is abused and ventricular fibrillation or asystole may occur. This cardiac stimulation has been implicated in the recent rise in asthma mortality thus indicating a need for a drug that has little β_1 activity. In addition, isoproterenol is a comparatively short acting bronchodilator because it is rapidly metabolized by the enzyme catechol-O-methyl transferase (COMT). Substitution of other groups or rearrangements of existing groups in the benzene ring have resulted in compounds more resistant to COMT with greater duration of action, and changes in the side chain have increased their specificity for β_2, or bronchial, as opposed to the β_1, or cardiac, receptors. The clinical, chemical and pharmacological considerations of the newer β adrenergic stimulants have recently been reviewed[7]. Examples of drugs claimed to have more selective β_2 stimulant actions than isoproterenol are; salbutamol[8], terbutaline[9], Th 1165a[10], hexoprenaline[11],, clorprenaline[12], trimethoquinol[13], quinterenol[14], WG 253[15] and soterenol[16]. Structure activity relationships of saligenin analogs of sympathomimetic catecholamines and the resolution of isomers of the most prominent of these analogs, salbutamol, have recently been reported[17,18].

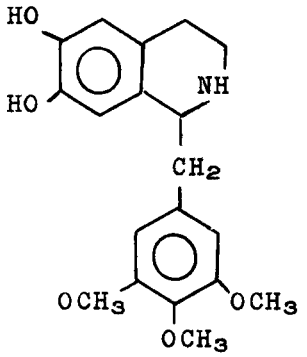
Clinically some of these selective adrenergic bronchodilators have succeeded in demonstrating less cardiovascular



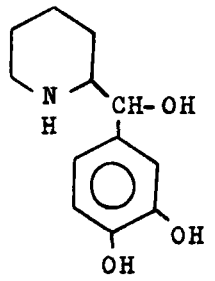
	R_1	R_2	R_3
Isoproterenol	3-OH	4-OH	$CH(CH_3)_2$
Salbutamol	3-CH_2OH	4-OH	$C(CH_3)_3$
Terbutaline	3-OH	5-OH	$C(CH_3)_3$
Soterenol	3-$NHSO_2CH_3$	4-OH	$CH(CH_3)_2$
Clorprenaline	2-Cl	H	$CH(CH_3)_2$
Metaproterenol	3-OH	5-OH	$CH(CH_3)_2$
Th 1165a	3-OH	5-OH	$CH(CH_3)CH_2C_6H_4p$-OH
Hexoprenaline	3-OH	4-OH	$(CH_2)_6NCH_2CH(OH)C_6H_3$-3,4$(OH)_2$



Quinterenol



Trimethoquinol

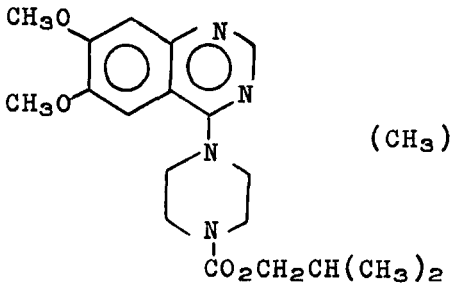


WG 253

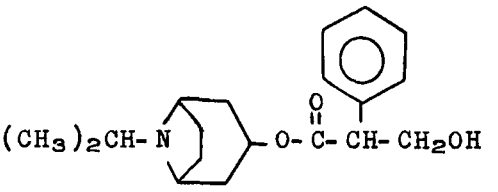
effects than isoproterenol. These drugs are effective as aerosols and unlike isoproterenol, they are also effective orally. Salbutamol[19-29] has been compared in double blind studies with isoproterenol[30-36], or metaproterenol[32,37-39]. At 0.2 mg, it is equiactive to isoproterenol and has a longer duration of activity. Terbutaline has demonstrated potent bronchodilator activity[40,41] when given orally (5 mg) or as an aerosol (0.2 mg). It has been compared to proxyphylline[42], isoproterenol[43] and metaproterenol[44-47]. Th 1165a aerosol at 0.2 mg is four times as potent as its analogue metaproterenol and is a relatively safe bronchodilator[48-50]. Hexoprenaline is effective as an aerosol (0.4%) or orally (0.5 mg), has a long duration of activity[11,51-53], and allows for the reduction of corticosteroid requirement[54,55]. Oral doses of trimethoquinol (1 mg)[56,57] and quinterenol (0.4 mg)[58] and aerosol doses of WG 253 (1.5 mg)[15,59] and soterenol (0.225 mg)[60] have bronchodilator efficacy equivalent to isoproterenol with a greater duration of activity and less β_1 stimulation. Clorprenaline has β_2 bronchodilator properties in dogs[12] and rats[61]. Sympathomimetic amines can also inhibit the release

of pharmacological mediators of the immediate type allergic reactions[62,63]. In a series of test systems including passive cutaneous anaphylaxis (PCA) and dextran response in the rat, isoproterenol was at least 1000 times as potent as disodium cromoglycate (DSCG)[64]. Therefore, β adrenergic stimulants are also potent protective agents against anaphylactic reactions and should not be considered only agents producing sympathomimetic relief of bronchospasm in asthma.

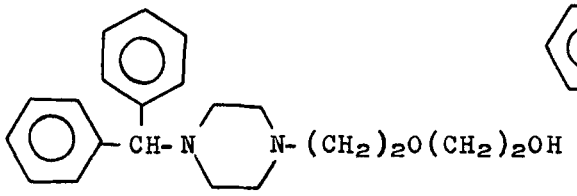
Non-Adrenergic Bronchodilators - Several new bronchodilators have been reported which do not depend on adrenergic stimulation as their mode of action. Piquizil, 4-(6,7-dimethoxyquinazolin)-4-piperazine-1-carboxylic acid isobutyl ester, a new oral bronchodilator, has been shown to be equal in activity to a combination of theophylline, ephedrine and phenobarbital[65]. Sch 1000, an atropine derivative, decreases pulmonary resistance in obstructive pulmonary disease when inhaled as an aerosol (0.025%)[52,66]. Decloxizine, a dechlorinated derivative of hydroxyzine, has marked antiserotonin and papaverine-like properties and is claimed to be an effective oral bronchodilator at 50 mg[67-69]. Fenspiride, an alpha adrenergic blocking drug, has been claimed to be of value in the treatment of asthma[70-73].



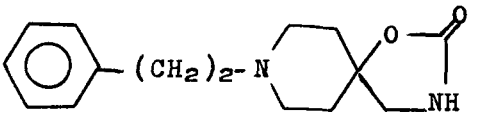
Piquizil



Sch 1000



Decloxizine

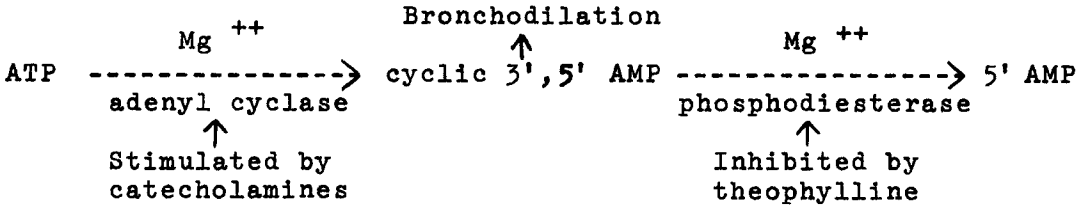


Fenspiride

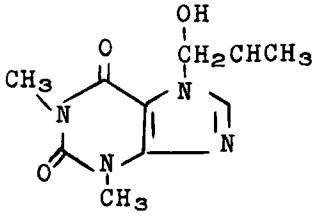
Prostaglandins - PGE_1 and PGE_2 are effective bronchodilators, capable of reversing the increased airway resistance and decreased airway compliance induced by cholinergic stimulation in the cat. Administration by aerosol produces the greatest bronchodilating response with little or no cardiovascular effects of hypotension and tachycardia. The cardiovascular effects were most pronounced after intravenous administration.

PGE_2 bronchodilator effects were not blocked by bilateral vagotomy, adrenalectomy, beta adrenergic or ganglionic blockade. These agents may represent a new and novel class of potent bronchodilator agents[74].

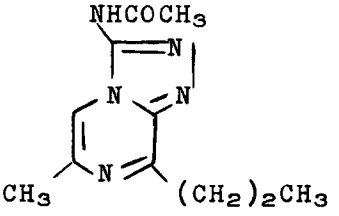
Phosphodiesterase Inhibitors - Catecholamines increase the concentration of cyclic AMP by stimulating the enzyme adenyl cyclase. Methylxanthines increase the concentration of cyclic AMP by interfering with its inactivation by phosphodiesterase and this is thought to be the biochemical basis for the bronchodilator action of theophylline.



ICI 58 301 (3-acetamido-6-methyl-8-n-propyl-s-triazolo [4,3-a] pyrazine) is a new compound that is a greater inhibitor of phosphodiesterase than theophylline. In animals, ICI 58 301 is far more powerful in preventing bronchospasm than in acting as a bronchodilator. This activity has been confirmed in asthmatic patients[75]. Proxyphylline, 7-(2-hydroxypropyl) theophylline, administered as a suppository (500 mg) is effective in enhancing pulmonary function in chronic asthmatic patients[76].

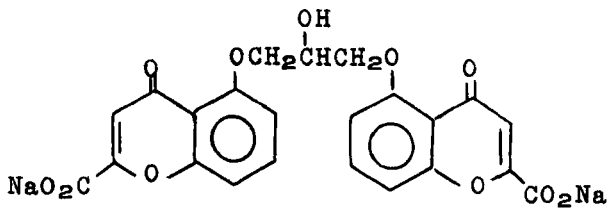


Proxyphylline



ICI 58 301

Disodium Cromoglycate (DSCG) - This compound appears to block a step in the chain of events triggered by the union of antigen with reaginic antibody which leads to the release of spasmogens and other mediators of the antiphylactic reaction[77] and has been found useful in treating asthma and allergic rhinitis. It differs from existing therapy in that it has no sympathomimetic, antiinflammatory or antihistaminic activity. DSCG is administered by inhalation as a dry powder in a special insufflator which permits controlled dosage. In a dose range of 20-80 mg daily, it has been used in the treatment of

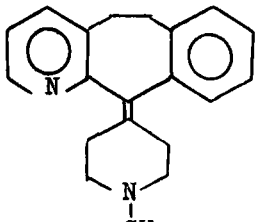


Disodium cromoglycate (DSCG)

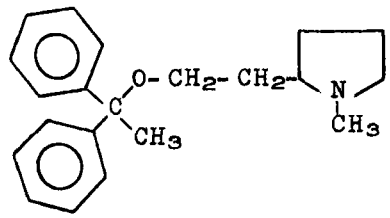
allergic rhinitis and has been found to be effective by some[78-80] and ineffective by others[81-83]. In extrinsic asthma in children, it has been found to be effective by most investigators[84-92] and allows for a decrease in the requirement of steroids[93-95] and bronchodilators[94,96]. DSCG can prevent asthmatic attacks[94,97], reduce histamine sensitivity[98,99] and exercise-induced airway obstruction[100] in asthmatics. The only side effect reported has been a pharyngeal and tracheal irritation[101,102] and although there is no evidence of drug-dependence there may be a loss of efficacy after some months of use[103].

Antihistamines - Antihistamines block histamine at the receptor site and are useful in the treatment of urticaria and rhinitis. Unfortunately, the majority of antihistamines produce an increasing degree of sedation with increased therapeutic activity.

Two new antihistamines, meclastine[104] and azatadine[105], have been reported to be clinically effective. Clinical studies show that azatadine is a potent, long acting antihistamine with low sedative liability at doses of 1-2 mg twice daily[106,107]. Azatadine, at 0.009-0.0024 mg/kg p.o., has been reported to be more potent than cyproheptadine as an antihistamine and antianaphylactic agent in mice and guinea pigs. It is generally less toxic than cyproheptadine and possesses significant anticholinergic and antiserotonin activity[105].



Azatadine



Meclastine

It has long been accepted that histamine is formed by the enzymatic decarboxylation of histidine. A drug that inhibits histidine-decarboxylase would decrease the amount of

histamine available and thus be of use in the treatment of allergic disorders. Tritoqualine has been reported to have non-specific antispasmotic and antiinflammatory activity besides inhibiting histidine-decarboxylase[108]. It has been shown to be effective in the treatment of hay fever and unlike most antihistamines, it has no sedative effect[109].

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Section III - Chemotherapeutic Agents

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Chapter 10. Antibiotics

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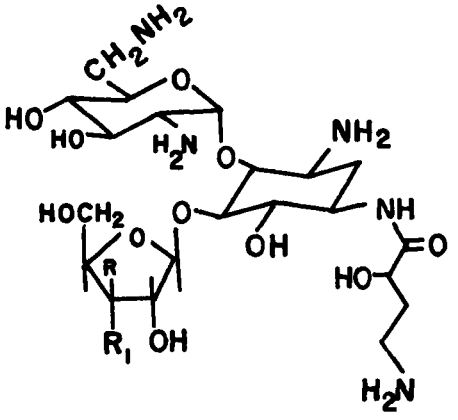
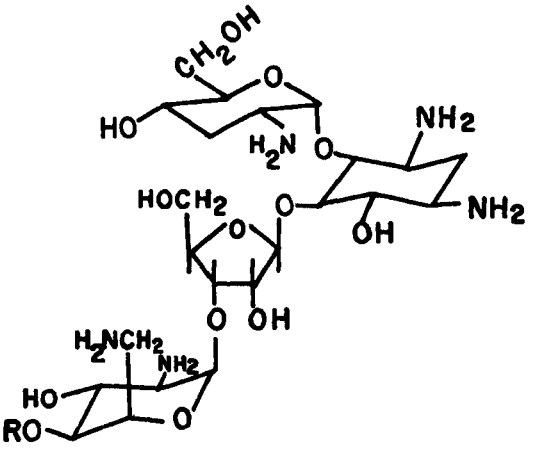
General - A symposium commemorating thirty years of penicillin therapy was held in May surveying presently active areas of scientific research in the penicillin field.[1] Commercial 6-APA processes,[1a] chemical relationships between penicillins and cephalosporins,[1b] mechanism of action,[1c] bacterial resistance,[1d] and immunological properties[1e] of penicillins were discussed, as were current clinical aspects of penicillin therapy.[1f] The physicochemical properties and biological activities of clinically significant β-lactam antibiotics were reviewed with particular commentary on β-lactamases.[2] A chapter on the chemistry of cephalosporin antibiotics appeared.[3] The Second International Symposium on Gentamicin brought up to date scientific and clinical information relating to this antibiotic, placed gentamicin in perspective among other aminoglycosides and defined areas in which further investigative efforts are needed.[4] Structure-activity relationships among chemically and microbially modified lincomycin antibiotics were reviewed.[5] A treatise on bacterial resistance to the macrolide antibiotics and lincomycins was published.[6] The chemistry, biological activity and biosynthesis of the ansamycins were reviewed.[7] Detailed accounts of totally synthetic approaches to the tetracycline antibiotics appeared.[8] A monograph on the biochemistry of antimicrobial agents has united significant developments in this area and orients readers to major literature reviews up to 1971.[9] A theory on the formation of antibiotics was proposed.[10] A symposium[11] held in March surveyed recent developments in many areas of the vast field of antibiotics.

Aminoglycosides - Comparative *in vitro* evaluations of tobramycin (nebramycin factor 6) continue to indicate it is two to four times more active than gentamicin against clinical isolates of *Pseudomonas aeruginosa*;[12-15] mean MIC values of 0.42 and 3.54 γ/ml, respectively, were reported.[12] Conversely, gentamicin proved four times as potent as tobramycin against isolates of *Serratia marcescens*;[14] both antibiotics demonstrated comparable activity against the majority of other gram-negative pathogens.[12-15]

Detailed accounts of the structure elucidations of the gentamicin C components[16-18] and sisomicin[19,20] were published. Chemical syntheses of 3'-deoxykanamycin,[21] 3',4'-dideoxykanamycin B[22] and 3',4'-dideoxyneamine[23] were announced along with preliminary biological data indicating their *in vitro* activity against *Escherichia coli* and *Ps. aeruginosa* strains which inactivate the parent antibiotics by 3'-O-

phosphorylation. Among new naturally occurring aminoglycosides, structures were proposed for butirosins[24-26] A(1a) and B(1b) and lividomycins A(2a)[27,28] and B(2b).[29] The butirosins, which were isolated from a bacterial fermentation, represent the first reported examples of aminoglycosides containing an N-acylated-2-deoxystreptamine moiety; an account of their biological profiles has not been reported. The lividomycin structures revealed their chemical immunity to enzymatic inactivation by 3'-O-phosphorylation, but O-phosphorylative inactivation of lividomycin A by strains of Ps. aeruginosa has been observed[30] and apparently occurs at a site other than the 3'-position.





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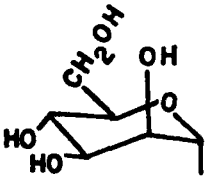
2a = R = 

1b = R = H, R_1 = OH

2b = R = H

Enzymatic inactivation of gentamicin by cell-free extracts from clinical isolates of Ps. aeruginosa[31,32] and R-factor carrying Klebsiella pneumoniae[33,34] was described. The mechanism of this pseudomonas resistance to gentamicin was established by showing that acetylation occurs on the 3-amino group of the 2-deoxystreptamine ring.[32] All gentamicin C components and sisomicin were reported to be excellent substrates for the inactivating enzyme gentamicin acetyltransferase, whereas tobramycin and kanamycin B proved to be poor substrates. A number of gentamicin-resistant tobramycin-sensitive clinical isolates of Ps. aeruginosa from various parts of the world have been studied and claimed to contain gentamicin acetyltransferase.[32] The klebsiella resistance to gentamicin was

reported to occur *via* adenylylation and further the presence of an R-factor which mediates gentamicin resistance in these klebsiella strains was demonstrated by transfer of this resistance *in vitro* to *E. coli* recipients subsequently designated *E. coli* JR66/W677 and ML1410 R^{+}_{100}.[33,34] A tentative structure for gentamicin C_1 adenylate has been proposed.[35] *E. coli* JR66/W677 has also been shown to adenylylate 3',4'-dideoxykanamycin B[36] and the structure of the inactivated product established as 3',4'-dideoxykanamycin B-2"-adenylate.[37]

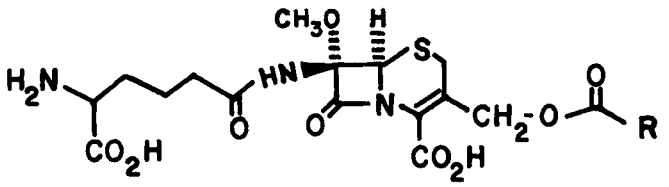
A report presenting clinical and laboratory evidence for the inactivation of gentamicin by carbenicillin appeared.[38] However, chemical,[39] kinetic[40] and clinical[41] data was published indicating that although gentamicin and carbenicillin can mutually inactivate each other, the reaction requires conditions that are not present in most patients requiring these agents.

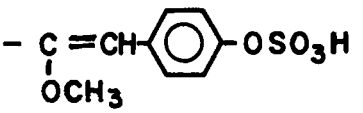
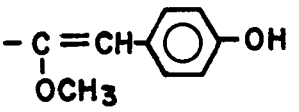
Spectinomycin became available in the U.S. for single dose treatment of acute gonorrheal infections; clinical studies indicate IM administration of 2.0 or 4.0 g of spectinomycin results in cure rates of over 90% in males and 96% in females.[42,43]

Ansamycins - Rifampicin became available in the U.S. during 1971 for use in the treatment of tuberculosis. Reviews containing discussions of clinical findings, antimicrobial activity and pharmacology of rifampicin were published.[44,45] Thrombocytopenia, apparently arising *via* an immunological mechanism, has been reported as a potentially serious side effect resulting from high-dose (1200 mg) twice-weekly rifampicin therapy.[46] The structure of streptovaricin C was revised in accord with results of new periodate oxidation experiments[47,48] and X-ray crystallographic studies.[49] Gross structures for six other naturally occurring streptovaricins were proposed.[50] The antiviral properties of the ansamycins are discussed in Chapter 12.

β-Lactams - Announcements of the discoveries of four novel naturally occurring cephalosporin C variants highlighted the year's developments in β-lactam research.[51-54] The new cephalosporins are uniquely substituted at positions C_3 and/or C_7 (cf. Table I) and were isolated from *Streptomyces* fermentations. In general, biological profiles suggest 7-methoxycephalosporins are more active than cephalosporin C against most gram-negative bacteria, but show poor activity against *Ps. aeruginosa*; they are essentially inactive against penicillin-resistant staphylococci. An elegant tranacylation process for cleaving the aminoadipoyl moiety of *5* with simultaneous introduction of new acyl side chains was reported.[55] Synthetic routes to 7-methoxycephalosporins and 6-methoxypenicillins which depart from benzhydryl 7-diazocephalosporanate and benzyl 6-diazopenicillinate, respectively, were described.[56] In related work, 7α-methyl-7-phenoxyacetamido cephalosporanic acid and methyl 6α-methylbenzylpenicillinate were synthesized as close mimics of the *D*-alanyl-*D*-alanine end of acetylmuramylpentapeptide, but were reported to be substantially less active than the parent compounds against gram-postive organisms.[57]

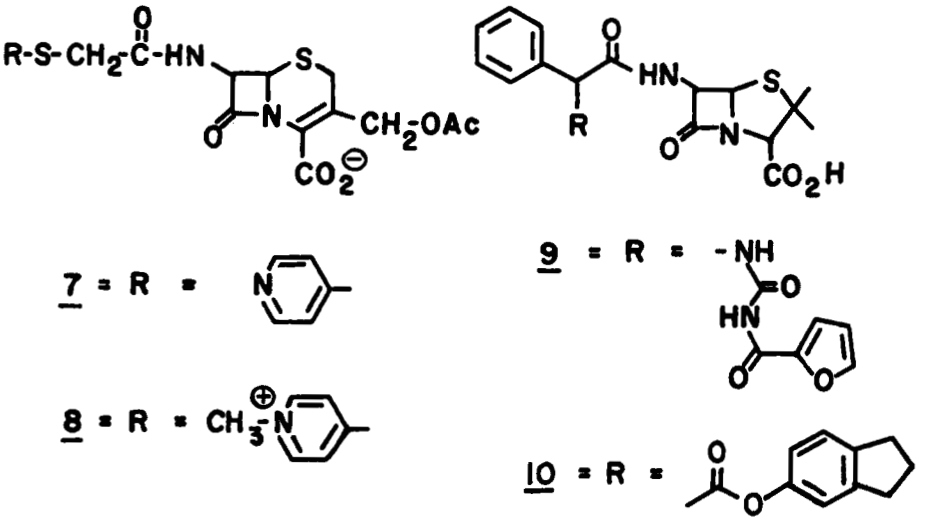
TABLE I



		R
3 = Cephamycin A	=	
4 = Cephamycin B	=	
5 = Cephamycin C (A16886B)	=	$-NH_2$
6 = A16884	=	$-CH_3$

Cephalexin (Keflex^R) has proven to be safe and effective in treating a variety of infections commonly encountered in general practice.[58] It is reported[59] that successful therapy of urinary tract infections with cephaloglycin (Kafocin^R) must be mainly attributed to the antibacterial activity of its metabolite desacetylcephaloglycin which is 4-8 times less active against gram-negative bacilli than cephaloglycin itself. Orally administered acetoxymethyl and pivaloyloxymethyl esters of cephaloglycin were reported to be enzymatically cleaved following absorption from the gastro-intestinal tract and to afford higher blood and urine levels than an equivalent dose of cephaloglycin.[60] Cephapirin (BL-P1322) (7), a parenteral cephalosporin, has antibacterial activity equivalent to cephalothin.[61-63] IM injection of 1 g of cephapirin sodium gave serum levels of 7.2-25 γ/ml and urine concentrations of 1-2.5 mg/ml;[61] an 88% cure rate was reported for urinary tract[64] and respiratory[61] infections caused by susceptible organisms. Less pain and phlebitis following i.v. administration is reported to be the major advantage of cephapirin over cephalothin.[61] BL-S217 (8) is claimed[65] to be 20 times more effective than cephalothin against Streptococcus pyogenes and Diplococcus pneumoniae infections in mice and 3-4 times more efficacious in an E. coli infection. Structure-activity relationships among a group of 7-[α-(N,N'-substituted-amidinothio)-acetamido cephalosporanic acids were described.[66]

Among semi-synthetic penicillins, BL-P1597 (9) and BL-P1654 were reported to be more active than carbenicillin against Pseudomonas, inhibiting 90% of 150 isolates at 12.5 γ/ml; conversely, carbenicillin was more active than both compounds against indole-positive proteus.[67] Sulfocillin was reported to have essentially the same in vitro spectrum and potency as carbenicillin.[68]



Orally administered indanyl carbenicillin (10) is absorbed and hydrolyzed in animals and man to provide carbenicillin activity generally equivalent to parenteral carbenicillin;[69] the metabolism of 10 in rats, dogs and humans was reported.[70] Satisfactory clinical results have been reported for indanyl carbenicillin therapy of urinary tract infections caused by Pseudomonas and Proteus species.[71-73] Results of a multi-center clinical evaluation of epicillin indicate this orally active ampicillin congener was generally effective in 84% of 683 patients with respiratory, gastro-intestinal or soft-tissue infections caused by a variety of ampicillin-sensitive gram-positive and gram-negative bacteria.[74]

Chemical properties of penicillin polymers derived from ampicillin, 6-APA and benzylpenicillin on standing in aqueous solution were described.[75] These polymers have been shown to combine covalently with protein and to elicit immune responses.[76] An enzymatic synthesis of ampicillin from 6-APA and various phenylglycine esters in the presence of Kluyvera citrophila KY-3641 was described.[77] Evidence has been published suggesting that the physiological effects of penicillin on E. coli may be due to interaction with at least two specific targets, both of which are

enzymes.[78] The application of multiparameter regression analysis techniques to the design of semisynthetic penicillins was discussed at length.[79] A monograph concerned mainly with chemical reactions and physiological properties of natural and synthetic β-lactams was published.[80]

Lincomycin/Clindamycin - The clinical efficacy of clindamycin[81] and lincomycin[82] against infections caused by nonspore-forming anaerobic bacteria was announced. Clindamycin was reported to inhibit 96% of 257 Bacteroides clinical isolates at 3.1 γ/ml or less.[83] A quantitative structure-activity study among lincomycin/clindamycin analogs suggests that the relative potency of members of these two sets of compounds depends only on the lipophilic nature of the pyrrolidine ring and not that of the molecule as a whole.[84] Relative enzymatic hydrolysis rates for different positional and structural esters of lincomycin in dog serum and simulated intestinal fluid were determined as part of a search for a lincomycin congener with superior pharmaceutical and kinetic properties; in vivo activity correlated with hydrolysis rates but no gross differences in esterase specificity were observed between dog serum and simulated intestinal fluid.[85] Microbial transformations of clindamycin[86,87] and N-demethylclindamycin[88] by Streptomyces species, in general, lead to pro-drug forms of the parent antibiotics.

Macrolides - Stereochemical problems in the macrolide area were reviewed in depth.[89] Structures and interrelationships between the leucomycins, maridomycins, espinomycins, SF-837 and YL-704 macrolides were summarized.[90] Two reports on syntheses of erythromycylamine indicate the 9-aminoerythromycin congener can now be conveniently prepared in kilogram quantities.[91,92] A series of chalcomycin analogs were described several of which demonstrated in vitro and in vivo activity against staphylococci comparable to the parent antibiotic.[93] Narbomycin aglycone, narbonolide, was isolated from a Streptomyces fermentation and its biogenetic relationship to narbomycin and picromycin established.[94]

Peptide antibiotics were reviewed with respect to biosynthesis[95] and the relationships between primary structure, conformation and antibacterial activity.[96] The elusive structure of viomycin was further resolved.[97] However, some doubt concerning the peptide sequence remains owing to an X-ray structure determination on closely related antibiotic tuberactinomycin O.[98] The relationship between viomycin and capreomycins 1A and 1B was established.[99] Tuberactinomycin N was reported to be as active as viomycin against experimental tuberculosis infections in mice and less ototoxic than both viomycin and capreomycin in guinea-pigs.[100] The structure of negamycin, a novel broad-spectrum hydrazide antibiotic, was determined.[101] An improved synthesis of gramicidin S via solid-phase synthesis and azide cyclization was carried out in 43-51% overall yield.[102]

Tetracyclines - The disposition of doxycycline by man, dog and rats was investigated.[103,104] Results in dog and rats indicate doxycycline passes relatively easily through the intestinal wall and is less

dependent upon normal renal function for its elimination than other tetracyclines of lesser lipophilicity; these findings are cited to help explain the clinical observation that doxycycline unlike other tetracyclines does not accumulate in patients with renal insufficiency.[105,106] Reports on structure activity relationships among tetracycline antibiotics derived by quantum mechanical calculations[107] and microbial kinetics[108] were described. An improved synthesis of minocycline (Minocin^{R}) utilizing 9-nitro-6-demethyl-6-deoxytetracycline was reported.[109]

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Chapter 11. Antifungal Agents

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Reviews - A review of the treatment of mycoses by use of antibiotics including griseofulvin, nystatin, amphotericin B, pimaricin, 5-fluoro-cytosine, trichomycin, cycloheximide, clotrimazole and miconazole, also covers the source of the antibiotic, chemistry, in vitro effects, clinical dosages, together with indications and side effects.[1] A recent text,[2] while not specifically related to antifungal agents presents aspects of mycology, of preservation techniques and detailed descriptions of methods related to diagnosis and culture of dermatophytes and subcutaneous and deep seated mycoses.

Methods - A new animal model for acute oral candidiasis in rats may provide an additional system for the preclinical evaluation of new antifungal agents.[3] Experimentally induced blastomycosis and histoplasmosis tend to mimic natural infections and are susceptible to amphotericin B therapy.[4] Use of analogue-resistant mutants to increase levels of end products, has been directed towards biosynthesis of pyrrolnitrins.[5] Strains were utilized which were resistant to 5-fluoro-tryptophane and 6-fluoro-tryptophane. These studies suggest a possible biosynthetic pathway for pyrrolnitrin in which D-tryptophane and reduced pyrrolnitrin are intermediates; 3-chloroindole appears to be a by-product since it markedly inhibits pyrrolnitrin synthesis.[5] The currently used procedure of cutting out antibiotic producing colonies from the agar in plugs, and placing them onto bioassay plates has been modified for kasugamycin producing strains. In the "agar piece method" the colonies are cut out prior to heavy growth, preventing extensive antibiotic diffusion.[6]

β-Alanine has been shown to increase the yield of antifungal antibiotic AYF produced by Streptomyces aureofaciens.[7] UV light and X-ray generate low-producing mutants as well as high producing mutants of Streptomyces nigrifaciens, with reference to antifungal production.[8] Nystatin has been successfully used in the selection of amino acid auxotrophs of Saccharomyces.[9] Neutron activation analysis for determination of chlorine has been utilized for an assay of griseofulvin in capsules and in tablet form.[10]

Clinical Experience - Intravenous administration of amphotericin B continues to be the major therapy for the treatment of systemic mycoses. Nebulization of amphotericin B was used to avoid some aspects of toxicity associated with intravenous use. Nebulization produced an aerosol with 80-90% of the drug

particles smaller than 8 microns, a diameter which should be retained in the peripheral bronchi. The successful treatment of 4 patients with coccidioidomycosis by this method, without toxicity, is reported.[11] Extracorporeal dialysis has been successful in extracting amphotericin B after intravenous administration in dogs, and in 3 patients with acute renal insufficiency.[12] The successful treatment of a case of primary aspergilloma of the cheek due to Aspergillus flavus with intravenous amphotericin B has been reported.[13] Rapid intravenous infusion of amphotericin B has been shown to produce higher peak serum levels of amphotericin than slow intravenous infusion, without greater toxicity.[14]

The enhancement of fungal keratitis seen with steroid anti-inflammatory agents was not observed in experimental Aspergillus keratitis of the cornea in rabbits when low levels of dexamethasone were combined with pimaricin. It appears that this topical combination may be effective and also avoid the aggravation of fungal keratitis observed with the steroids alone.[15] Pimaricin topically, in combination with the antitrichomonal agent metronidazole orally, appears useful in the elimination of Trichomonas and prevention of the Candida overgrowth which frequently follows antitrichomonal therapy.[16] Topical pimaricin therapy of Candida vaginal infections and mixed Candida and Trichomonas infections yielded satisfactory results in approximately 80% of 286 women.[17]

Several additional clinical reports have appeared on the use of 5-fluorocytosine. The successful therapy of Cryptococcus meningoencephalitis when the drug was given at a level of 6 grams per day for 45 days has been reported. Relapse occurred after withdrawal of the drug; cultures became positive and additional therapy was judged unsuccessful.[18] Several other documented relapses while patients are under therapy with 5-fluoro-cytosine for cryptococcal meningitis have been described,[19] and, in some cases have been attributed to development of resistance during therapy. Oral doses of 2 to 5 grams a day of 5-fluorocytosine have been successful in reducing urinary counts of Candida in some instances, and have apparently eliminated urinary candidiasis in other cases.[20] Serum binding of 5-fluorocytosine was calculated to be approximately 50%; however the fungicidal effect of 5-fluorocytosine on Candida was enhanced in vitro by the addition of serum to the media. Urine appeared to inhibit the in vitro action of 5-fluorocytosine.[20]

Clotrimazole (Bay b 5097) has been the object of additional clinical and laboratory evaluation. In children with C. albicans pyelonephritis who were dosed at a level of 70-200 mg/kg/day for 10 to 40 days, good response was noted in all patients although relapses frequently occurred after the

cessation of therapy.[21] Contrary to initial reports, a recent study found a number of C. albicans strains which were not sensitive to obtainable serum levels of clotrimazole.[22] Further investigations have shown Candida to be more sensitive in vitro than Cryptococcus.[23] The in vitro activity of clotrimazole against Blastomyces, Histoplasma, Sporothrix, Cryptococcus and Coccidioides was shown to be comparable to amphotericin B, while its activity against Candida and Aspergillus was less than that of amphotericin B. Against dermatophytes, clotrimazole had activity comparable to pyrrolnitrin[24] but less than tolnaftate[25] and greater than that of griseofulvin.[24,25] Some in vitro activity against gram-positive bacteria has also been demonstrated.[25] In guinea pigs experimentally infected with Trichophyton mentagrophytes, topical therapy with clotrimazole was less successful than with tolnaftate; oral therapy gave results similar to that seen with griseofulvin.[25] In experimental infections in mice infected with Aspergillus, Blastomyces or Candida, therapy with clotrimazole was unsuccessful, however some protection was obtained in Cryptococcus and Coccidioides infections at high oral dose regimens.[26] A drug induced mechanism for in vivo drug inactivation has been described.[25,26]

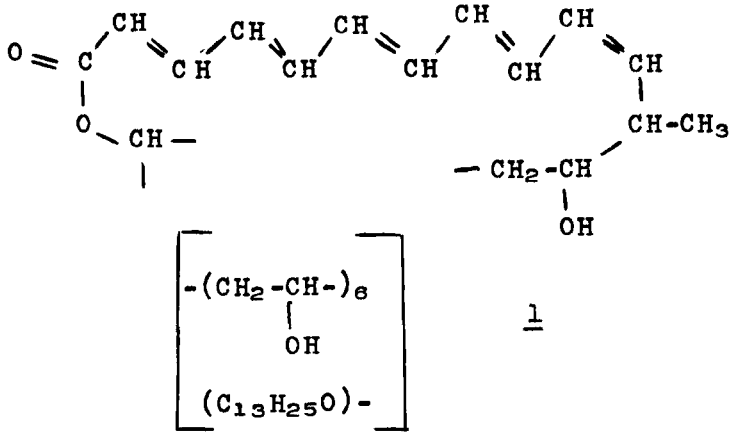
Thiabendazole, at 25 mg/kg/day orally for 1.5-22 months, appeared successful in 1/3 of 14 patients with chromomycosis. However, hypersensitivity reactions manifested by elevated serum bilirubin and transaminase levels developed in several patients.[27]

Griseofulvin continues to be the main oral therapy for a number of dermatophyte infections, and attempts to improve the speed and degree of absorption of griseofulvin following oral administration have continued. Dispersions of griseofulvin in a solid matrix of polyethylene glycol 6000, was demonstrated to lead to higher serum levels in man than those obtained with commercial micronized griseofulvin.[28] The vasodilator effect of griseofulvin has been utilized in the treatment of Raynaud's Phenomenon.[29,30] The use of dimethyl sulfoxide as a vehicle for griseofulvin has been shown to increase the penetration of griseofulvin into the subkeratinous tissue. Topical therapy with griseofulvin in DMSO was successful in 10 cats with Microsporum canis.[31]

Miconazole nitrate, previously shown to be effective in topical treatment of Trichophyton infections, was effective when used topically for 1 to 3 weeks as a 2% cream in 44 of 46 cases of Candida vaginitis, but it was without effect in 2 cases of vaginal Torulopsis glabrata.[32]

New Antifungal Agents - Tetranactin, a new macrotetrolid antibiotic similar to nonactin and produced by a strain of Strep-

tomyces aureus shows in vitro activity against some fungi and gram-positive bacteria as well as insecticidal activity. The acute toxicity of tetranactin in mice is low.[33] Flavomycoin is a new polyene antifungal, isolated from Streptomyces roseoflavus, containing a pentaene system in conjugation with a lactone carbonyl group. It has the partial formula, 1.[34,35]



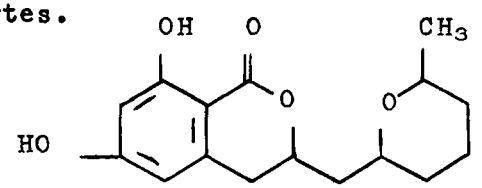
1

Tetramycin is another new polyene antifungal produced by a variety of Streptomyces noursei; it has an empirical formula $C_{34}H_{53}O_{14}N$ and good in vitro activity against a variety of yeasts and filamentous fungi. The acute intravenous LD_{50} of tetramycin in mice is 50 mg/kg.[36]

Three new antibiotics related to phleomycin and bleomycin have been isolated from fermentations of Streptomyces bikiniensis variety zorbonensis. These products have in vitro activity against gram-positive and gram-negative bacteria as well as various fungi. The empirical formulae for the antibiotics are: zorbomycin, $C_{56}H_{100}N_{28}O_{24}S_2Cu$; zorbonomycin B, $C_{58}H_{90}N_{18}O_{24}S_2Cu$; zorbonomycin C, has not been completely characterized but also contains Cu.[37] A new antibiotic, 24010, possible related to tunicamycin has activity against a variety of phytopathogenic fungi, Candida and Saccharomyces.[38]

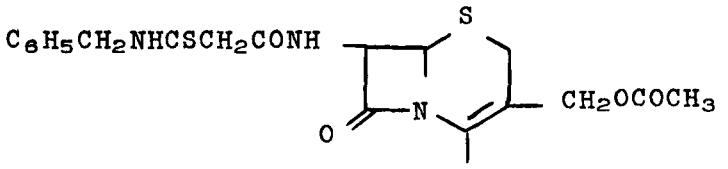
Prumycin, has an empirical formula $C_8H_{19}N_3O_5$; it is a new water soluble, basic antibiotic active against some bacteria and fungi and it is produced by a strain of Streptomyces.[39] Tunicamycin is a new antibiotic isolated from Streptomyces lysosuperificus which has activity against animal and plant viruses and gram-positive bacteria as well as yeasts and fungi.[40] Hikizimycin, produced by a new Streptomyces strain has some antibacterial as well as antifungal activity and appears to be the first example of an antibiotic with cytosine and kanosamine as substituents.[41] Cladosporin, 2, is a new antifungal metabolite produced by Cladosporium cladosporioides; it has modest in vitro activity against a variety of derma-

tophytes.

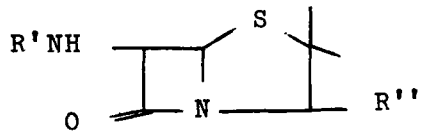


2

Amongst a series of semi-synthetic β-lactam compounds, the cephalosporin derivative, 3, and the penicillin derivatives, 4,5, showed unexpected antifungal activity in vitro; compound 3 was ineffective, however, in an experimental systemic Cryptococcus neoformans infection in mice.[43]



3

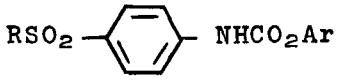


4 R' = $C_6H_5OCH(CH_3)CO$; R" = COSK

5 R' = $C_6H_5OCH_2CO$; R" = CHO

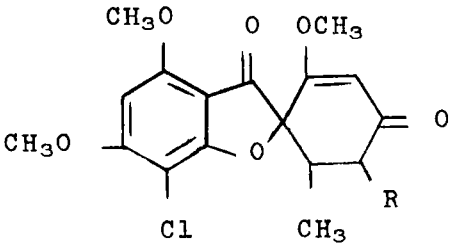
4, 5

A series of substituted carbamate esters (6-14), have in vitro



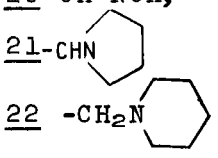
	R	Ar
6	CH_3	2,4,6-tricholorophenyl
7	CH_3	pentachlorophenyl
8	C_2H_5	pentachlorophenyl
9	p-FC_6H_4	pentachlorophenyl
10	H_2N	pentachlorophenyl
11	$(CH_3)_2N$	pentachlorophenyl
12	$(CH_2)_4N$	pentachlorophenyl
13	$O(CH_2)_4N$	pentachlorophenyl
14	$(CH_2)_5N$	pentachlorophenyl

activity against C. albicans and Aspergillus niger. The most active were 13 and 14. The corresponding trichlorophenyl derivatives of 8, 10, 12, 13 were inactive.[44] A series of griseofulvin analogues, derived from transformation of 5'-formyl griseofulvin, 15 through 24, have good in vitro activity against a number of dermatophytes; 24 was more active



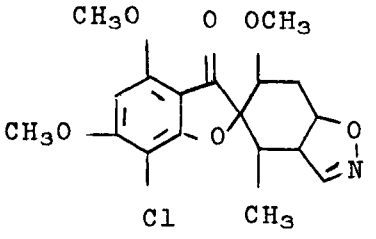
R =

15-SCH_3, 16=CH_2, 17 CN,
18-$NNHC_6H_5$, 19-CH=NN $(CH_3)_2$,
20-CH=NOH,



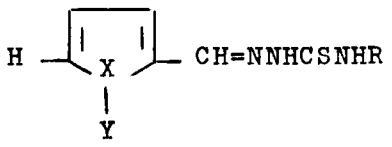
22 $-CH_2N$

23 $-CH_3$



24

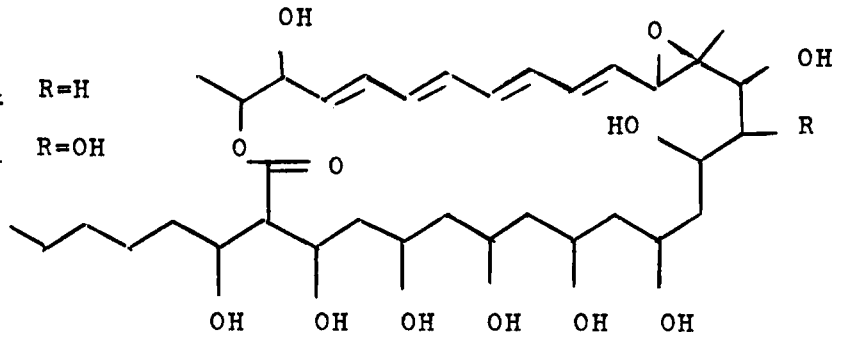
in vitro than griseofulvin. All were without activity in vivo except 20 which was considerably less active than griseofulvin against M. canis in guinea pigs.[45] A series of unsymetrical carbonyl disulfides, RC(O)SSR', were tested in vitro against Histoplasma capsulatum; the most active were those where both R and R' were short unbranched alkyl or unsubstituted phenyl moieties. No meaningful in vivo activity was observed in the series.[46] In a large series of thiosemicarbazones and 4-substituted thiosemicarbazones made from heterocyclic aldehydes, 25 through 30, have interesting in vitro activity.[47]



	X	Y	R
25	N	H	Ph
26	N	H	3-FC_6H_4
27	N	H	m-$O_2NC_6H_4$
28	N	H	Ph $(CH_2)_2$
29	O	CH_3	Et
30	O	CH_3	H

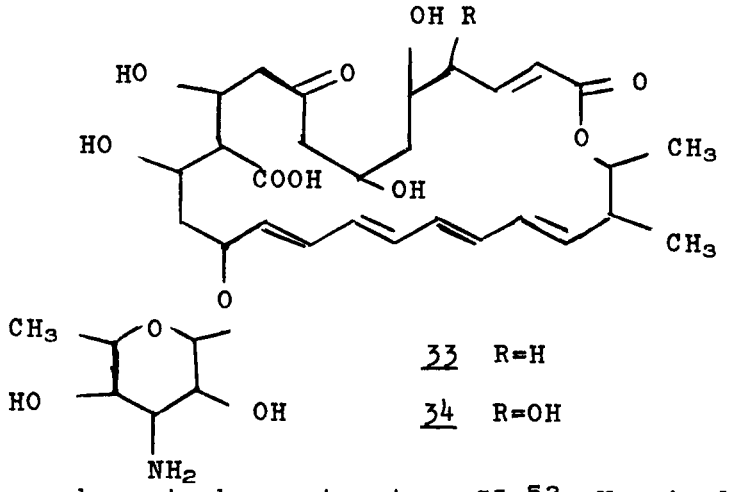
Chemical and Physical Studies of Antifungal Agents - The previous total synthesis of antimycin A_3 provided a diasteriomeric mixture. A total synthesis of natural antimycin A_2 has

now been reported.[48] The structures of the autooxidation products of filipin and lagosin have been shown to be the corresponding tetraene epoxides 31 and 32.[49] The polyenes



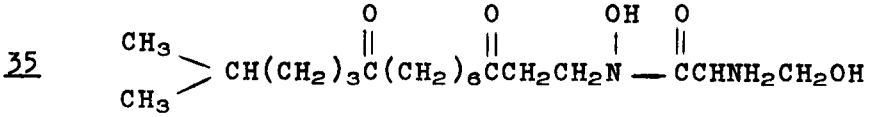
31 R=H
32 R=OH

tetrin A and tetrin B have been shown to have structures 33 and 34 respectively.[50,51] The structure of lipoxamycin has



33 R=H
34 R=OH

been shown to have structure 35.[52] Versicolin, an antifungal antibiotic is 2, 3, 6-trihydroxy-toluene, and this has been confirmed by synthesis.[53]



35

Linear free energy relations, correlating data from more than 30 structural types of antifungal agents with hydrophobic and electronic parameters have been formulated. In a number of these examples, the antifungal activity closely paralleled antibacterial and hemolytic activity, suggesting that these fungicides may bring about their action by membrane effects.[54]

Biological Studies of Antifungal Agents - The primary site of action of siccanin on T. mentagrophytes appears to be the succinate dehydrogenase system of terminal electron transport. This suggests that siccanin inhibits fungal growth by inhibition of the respiratory electron transport system.[55] The primary action of pyrrolnitrin against Microsporum gypseum has been shown to be blockage of the electron transfer between the flavo protein of NADH dehydrogenase and the cytochrome B of the respiratory chain.[56] The mode of action of yemenimycin against C. albicans is suggested to be interference with DNA synthesis.[57]

The polyenic antibiotic dermostatin which possesses in vitro activity against dermatophytes and Candida has been re-examined with a view towards its in vitro and in vivo activity against Cryptococcus, Blastomyces and Histoplasma in a variety of test systems. Dermostatin was less active in vivo than amphotericin B against C. albicans and H. capsulatum while the activities of the 2 antibiotics were comparable against C. neoformans and Blastomyces dermatitidis infections in mice.[58]

Saramycetin has been shown to retard the clearance of sulphobromophthalein (BSP) in man. This may be due to inhibition of the hepatic enzyme which conjugates BSP to reduced glutathione, or to the anticholeretic activity of saramycetin.[59] Amphotericin B has been shown to increase the permeability of frog skin to potassium, chloride and urea.[60] It has been suggested that the differential activity of griseofulvin against T. mentagrophytes and Trichophyton rubrum in vitro may be useful in the differential diagnosis of these two species.[61] In vitro studies with Saccharomyces, Candida and several strains of Cryptococcus have shown synergistic activity with a combination of amphotericin B and 5-fluorocytosine.[62] Natural resistance of Candida to nystatin and amphotericin B may not occur; however resistance can be developed in vitro to these agents as well as candicidin, pimaricin and filipin.[63]

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Chapter 12. Antiviral Agents

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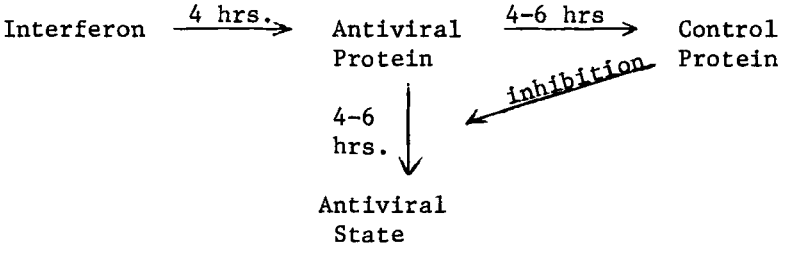
Introduction - Interest was high in several areas of antiviral research during the past year. As research continues it becomes clear that interferon is a complex story and most likely just one facet of a much larger immunological picture. No new non-polymeric interferon inducing drugs were discovered. A single human study with tilorone was reported. The role of RNA directed DNA-polymerase with regard to oncogenic viruses has been discussed[1] and a search for inhibitors of this enzyme as potential antiviral agents has focused on the ansamacrolides. Isoprinosine is claimed to have a range of antiviral activity in experimental laboratory infections and uncontrolled human trials claiming efficacy have been reported from Argentina. A notable advance is the discovery of a drug for the prevention of Marek's disease in poultry. Several reviews[2-6] discussed the state-of-the-art during 1971.

Interferon - Human interferon was shown to have a limited but measurable activity against a number of strains of adenovirus.[7] The absence of interferon[8] in serial serum specimens collected prior to and during the incubation and acute phase of transfusion associated hepatitis may in part be related to the chronicity of the disease. Human leukocytal interferon has been utilized in at least two controlled clinical trials. In the first trial[9] it was administered to patients with confirmed influenza and found to rapidly and dramatically reduce the systemic symptoms (headache, dizziness, malaise) and to a lesser degree the respiratory tract symptoms. The second study[10] utilized interferon prophylactically and was conducted with children in Moscow during the "flu" season. There was a 45-60% reduction in the incidence of influenza and respiratory infections. Production of interferon[11] in volunteers infected with either partly attenuated influenza A_2/Leningrad/65 or B/England/62 is greatest in those who develop most antibody. There was no correlation between amount of interferon produced and disease severity. The importance of antibody in an untreated infection has recently been demonstrated.[12] Normal mice given a sublethal dose of vaccinia virus produce interferon and antibody. Mice immunosuppressed by cyclophosphamide treatment or whole body X-irradiation do not produce antibody and exhibit 100% mortality after vaccinia challenge even though interferon is still formed. Administration of antibody causes a marked reduction in mortality. On the other hand immunosuppressed mice are protected[13] against lethal vaccinia infection when treated early enough with the interferon inducers poly I:C or Newcastle disease virus (NDV).

Other "factors" which resemble or are identical with interferon have also been identified. A serum protective factor (SPF) has been isolated[14] from mice infected with Langat virus. It was shown that SPF is not an immunoglobulin, but it is found in the globulin fraction, and it also differs from interferon in that it does not protect mice under 3 weeks of

age or neonatally thymectomized mice up to 6 weeks of age against Langat virus infection. The species specificity of SPF was not examined nor was its effect against other viruses tested. Poly IC was found to cause the formation of a colony inhibitor which appeared to be identical to interferon.[15] The interaction of sensitized mouse peritoneal or splenic leukocytes with L-cells was found to release inteferon.[16]

An experiment conducted with mouse-monkey hybrid cells[17] indicated that there were different genetic sites for the synthesis of interferon and the protein responsible for the antiviral state. This data is consistent with recent in vitro experiments[18] conducted with actinomycin D. In these experiments cells were incubated with interferon for different time intervals, the interferon removed, and the cells incubated with actinomycin for 3 hours and then challenged with vesicular stomatitis virus (VSV) and plaques counted. There was no antiviral state produced when actinomycin was added prior to interferon, but the antiviral state was achieved and potentiated some 30-fold when actinomycin was added at 4-5 hours and became maximal (100-fold) when actinomycin was added at 5 1/2-6 hours. These data are consistent with the following scheme:



Similar experiments have also been carried out using poly IC.[19] In further recent experiments[20] a tissue antagonist of interferon was isolated which may be the control protein, because when it was added at 4-6 hours under the same experimental conditions described above, it decreased by 40 fold the antiviral state induced by interferon. Its activity is blocked by actinomycin D. The protection against MM virus afforded by NDV, statalon, endotoxin, pyran copolymer, poly IC or interferon is also antagonized by estrone.[21]

Interferon Inducers

1) Polynucleotides - Low titres of interferon were seen in 14 out of 20 human subjects given intravenous poly IC in doses up to 4 mg/kg. The chief clinical feature was a febrile response and there were no abnormalities reported in any of the clinical parameters studied. Subjects given a second injection of poly IC gave an interferon response, whereas repeated daily injections caused a refractory state.[22] Experiments with mice indicate[23] that the refractory state can be overcome to some degree by following a strict dosage regimen of either 25 mcg. or 200 mcg. of poly IC every 12 hours for 6 days. This practice was shown to give sustained levels (hundreds of units) of interferon. With rabbits,[24] it was shown

that there was no correlation between interferon response and febrile response to poly IC. In addition, prior administration of indomethacin markedly reduced the elevation of body temperature but was without effect on the interferon stimulation by poly IC. In animal protection studies poly IC at 1 mg/kg either intravenously or intramuscularly[25] has been demonstrated to protect rabbits against up to 625 LD_{50} of rabies street virus, and to protect mice against Semliki Forest virus (SFV) infection[26] when treatment (100 mg intraperitoneally) was begun as late as 5 days after infection when high titers of virus were already in the brain. It did not prevent infection in calves challenged with infectious bovine rhinotracheitis (IBR) but it did ameliorate the clinical systems of respiratory illness, when administered at doses of 1 mg/kg intravenously and provided complete protection[27] from the pneumonic lung lesions of IBR. An attempted cure of a single infant with herpes type 1 encephalitis using a dosage of 1 mg/kg of poly IC for 11 days, was discussed.[28]

Further toxicological studies with poly IC have shown it to have no adverse effect on HFL-1B cells.[29] *In vivo* experiments indicate a depression of zinc and iron concentrations in the serum of rats and rabbits but not monkeys;[30] in acute and subchronic (42 days) studies using 2 mg/kg/day, poly IC was well tolerated by rodents but not by dogs;[31] in calves it caused[32] CNS depression and respiratory symptoms at doses of 0.1-1.2 mg/kg intravenously although at these doses very low titers of interferon were detected in the serum.

Effects of poly IC on viral oncogenesis have been widely studied and a review on the subject has appeared.[33] A recent study[34] using a double-stranded RNA of fungal origin dramatically indicates the types of responses that are seen in such experiments. Innoculation of mice with Friend virus results in a progressive increase in spleen size, followed by eventual rupture and death. Administration of double-stranded RNA prior to or in the early stages of infection increases the severity of the disease, whereas treatment with the RNA beginning 5 days after infection causes a marked reduction in spleen size and a more normal histological appearance of the spleen. The treated mice remained in remission for the 6 week duration of the experiment. The beginning of treatment at day 5 corresponds to the time of rapid proliferation of cells which cause the spleens to increase in size.

There have been some interesting probes into the structural requirements for activity with double-stranded polynucleotides. Greater *in vitro* antiviral activity than is found with poly IC can be obtained by the sequential addition of the single-stranded homopolymer constituents;[35] e.g. addition of poly I followed at 10 min or even 1 day by poly C causes enhanced antiviral activity as does the addition of poly C followed at 1 min or 10 min by poly I. *In vivo* the homopolymers must be injected intravenously within 1 minute of each other to achieve interferon levels even comparable with poly IC. This may well be a reflection upon the metabolic stability of single *vs* double-stranded RNA. Incubation of poly IC or even the alternating ribonucleotide copolymer poly r (A-U) at 37° in

Eagles minimal essential medium causes an enhancement of interferon inducing activity.[36] A systematic study[37] of poly IC in which the molecular weight of only one of the homopolymers was varied at a time leaving the other component of the duplex intact showed that antiviral activity and toxicity (mouse LD_{50}) decreased in parallel as the molecular weight of the variable molecular weight component was decreased. A convenient method for molecular weight determination of polynucleotides is also described.[37] A later study with poly C (I_{P5})I confirms these results.[38] Complexes of poly IC and DEAE-dextran are known to enhance the potency of the former. It was concluded[39] from a study of this effect using various concentrations of DEAE-dextran that optimal activity was achieved when the complex aggregates, and that this facilitates uptake by cells and protects against endonucleases. These same authors showed[40] that poly I-poly (1-vinylcytosine) is equivalent to poly IC in inducing interferon on human skin fibroblasts and postulated that its reduced charge/mass ratio and tendency to aggregate causes enhanced cellular uptake leading to activity.

Additional double-stranded RNAs were discovered to be interferon inducers. The activity of a DNA-RNA hybrid synthesized from single-stranded DNA of f_1 phage was demonstrated by complement fixation techniques to be entirely due to contaminanting double-stranded RNA.[41] Other active double-stranded RNAs were isolated from normal mammalian cells.[42,43] Finally, it is claimed that a double-stranded RNA equivalent in potency to poly IC, could be produced on large scale using a restrictive E. coli strain and a mutant f_2 phage.[44]

Statolon[45] administered intranasally protected mice against influenza, and was shown to produce interferon in the trachea and lungs. It was non-antigenic, and its protective effect lasted for at least 1 week.

2) Other Polymers - Chlorite oxidized oxyamylose (COAM)[46] was found to protect mice against foot and mouth disease virus but did not protect swine against the natural infection. In a hog cholera infection COAM provided no increase in survival time but did delay the onset of disease symptoms. Circulating interferon was not detected in swine treated with COAM. Although COAM given prophylactically to mice afforded some protection against influenza A/PR8, it was concluded[47] that it acts by triggering host response and not via interferon.

Phosphomannans isolated from several species of yeast[48] are a new class of relatively non-toxic (acute LD_{50}) interferon inducers. They cause formation of lower amounts of interferon in the rabbit than poly IC, but have similar induction kinetics. The materials retained their activity after digestion with beef pancreatic ribonuclease, thus ruling out the presence of RNA.

3) Tilorone - The bulk of the biological studies reported in preliminary form last year have now been confirmed by others.[49] Additional analogs possessing activity similar to tilorone were also described.[50] The first clinical experiments were described[51] this year. This study examined the

interferon response after administration of tilorone to the eyes of 10 subjects (1 drop of 200 mg/ml solution), and 1.0 g orally to 3 subjects; no interferon was detected by either route, and it was observed that the drug was deposited in the corneal epithelium. This deposit required up to 2 months after drug administration for complete disappearance. With the exception of some gastrointestinal irritation, no other adverse effects were noted. Tilorone did not induce circulating interferon in the chick, nor did it afford protection against Avian influenza.[52] In this test statolon was effective but poly IC was not.

Natural Products

Rifampicin when applied as a 10% ointment or 15% cream to the vaccination sites of human subjects prevented a positive reaction in about 50% of the cases.[53] With the use of the cream formulation a decrease in immune response was noted in all subjects as measured by seroconversion. There was no antiviral activity by the oral route. In animal experiments, oral rifampicin had no effect against herpes simplex, Mengo virus, intranasal influenza A/PR 8 or against intracerebral vaccinia infections of mice.[54] However, a significant dose-related reduction of tail lesions in vaccinia infected mice was demonstrated; the optimum regimen was 250 mg/kg of rifampicin beginning 24 hours prior to infection and continuing for 4 days.

In vitro studies[55] of anti-vaccinia activity have been carried out with a series of rifamycin derivatives and with their corresponding aminopiperazine side chains. Several of the side chains *per se* were claimed to be more active than the rifamycin. These effects could not be demonstrated by other workers.[56] It was shown by plaque assays and by morphological changes in cells, that the side-chain of rifampicin had no effect on the course of virus infection. Several mechanisms of antiviral action have been proposed[57,58] for rifamycin derivatives but the greatest interest centers around the inhibition of RNA-directed DNA polymerase. Several rifamycin derivatives but not rifampicin itself exhibit greater than 50% inhibition[59] of the RNA-directed DNA polymerase of Moloney sarcoma virus (MSV) at concentrations of 50-100 mcg/ml.

Rifampicin inhibits[60] focus formation by Rous sarcoma virus in chick embryo fibroblasts, but it was postulated that this effect was due primarily to the inhibition of cell proliferation that was also seen. A similar situation was described[61] for the 1-amino-2,6-dimethyl-benzylpiperazine derivative of rifamycin which, in addition to totally inhibiting focus formation and virus replication of MSV, was also found to reduce cell proliferation by 60%. With a more highly purified sample of drug, however, there was inhibition of viral replication and cell transformation, but there was no decrease in cell proliferation.

The streptovaricins, which are also ansamacrolides, appear to elicit effects similar to the rifamycins. Inhibition of focus formation by MSV was demonstrated[62] with streptovaricins at doses 4 times lower than the cytotoxic dose. It is speculated that the inhibition of cell transforma-

tion is due to inhibition of the viral DNA polymerase. Streptovaricin when administered at a dose of 1.5 g/kg/day in feed to leukemia virus infected mice, caused a 50% reduction in spleen weight.[63] Virus replication in the spleen was unaffected. Since there was no reduction of spleen weight in mice with a non-viral transplantable tumor (L1210) strain, it was reasoned that streptovaricin did not inhibit malignant cell proliferation and therefore presumably inhibited a virus specific event in cell transformation such as the reverse transcriptase.

Some miscellaneous natural products which have exhibited limited antiviral activity are filipin[64] *in vitro vs.* NDV; fusidin[65] *in vitro vs.* influenza A/PR 8; melanacidins[66] (from the 3,6-epidithiadiketopiperazine group) *in vivo vs.* herpes virus; tunicamycin[67] *in vitro vs* NDV; antibiotics from starfish[68] *in vitro vs* influenza; L-asparaginase[69] *in vitro* and *in vivo* (rabbit eye) *vs* vaccinia and myxoma; colchicine, demecolcine, vinblastine and vincristine[70] *in vivo* (rabbit eye) *vs* herpes simplex. A clinical trial with vitamin C[71] in 40 subjects challenged with a cold virus has been carried out but the results have not been published in the scientific literature.

Nucleosides - It appears that there is increasing use of cytosine arabinoside in varicella infections in leukemic children. A dramatic improvement in a disseminated varicella infection in a leukemic patient was achieved[72] with cytosine arabinoside administered daily by the intravenous route at doses of 100 mg/meter^2. Most recently, it was reported[73] that best results are obtained by continuous intravenous infusion at doses of 10-40 mg./meter^2. It was noted that if the patient survives for 48-72 hours there is a good response to the drug. High doses of cytosine arabinoside (100 mg/kg intraperitoneally) were demonstrated[74] in a toxicology study to cause malformation of embryos in pregnant rats.

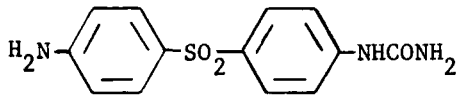
Reviews on the use of idoxuridine in herpes simplex[75] and herpes hominis[76] virus infections have been published.

Isoprinosine (NPT-10381) - It is proposed[77] that the anti-influenza activity of isoprinosine (the p-acetamidobenzoate salt of the 1:3 complex of inosine and dimethylaminoisopropanol) is due to an alteration of ribosomes such that they are more selective for host mRNA and less selective for viral mRNA. Isoprinosine administered to mice 24 hours after infection with influenza virus provided protection against mortality[78] and with sublethal virus innocula it decreased the respiratory morbidity. In experiments[79] with herpes, vaccinia, polio and echovirus there was no reduction in cytopathic effect but there was a reduction in virus yield. *In vivo* experiments[79] indicated a decrease in mortality from herpes corneal infection in hamsters and a reduction in tail lesions in vaccinia infected mice. No protection was afforded against encephalomyocarditis in the mouse.

A controlled study[80] of the effect of isoprinosine (1 g q.i.d.) in subjects with viral hepatitis indicated that hepatitis-associated antigen (HAA) positive patients do not respond to the drug, but HAA negative patients improved considerably (liver function parameters) relative to

the placebo group. Isoprinosine (4-6 g/day) was shown[81] to be of no value in the treatment of amyotropic lateral sclerosis. A large uncontrolled study[82] of isoprinosine (40 mg/kg/day in children; 50 mg/kg/day in adults) in influenza, rhinovirus, mumps and measles was claimed to ameliorate the systemic symptoms (fever, headache, malaise) and to reduce the time and intensity of the specific disease symptoms.

Marek's Disease - p-Ureido-p'-aminodiphenylsulfone when administered continuously in feed at a level of 0.0002% (1) was effective[83] in preventing the mortality and tumor development in birds infected with Marek's disease.



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Amines - Double-blind trials were conducted with cyclooctylamine hydrochloride administered intranasally for 2 days prior to and for 7 days following nasopharyngeal challenge with 64,000 TC ID_{50} of influenza A_2/HK. The drug was effective in reducing the severity of the disease as measured by reduced febrile responses, lower virus recovery and reduced serum antibody titer.

Structure-activity relationships within the amantadine series have been discussed.[84]

Miscellaneous Synthetics - Florenal (undisclosed structure) was shown to have in vitro and in vivo activity against herpes virus.[85] An uncontrolled clinical trial in a large number of patients claimed[85] dramatic results in the treatment of herpetic keratitis, adenovirus conjunctivitis and epidemic keratoconjunctivitis. 5-Fluoro-2-(α-hydroxybenzyl)-1-propyl-benzimidazole was reported[86] to be 10 times more potent than the unfluorinated parent against polio and coxsackie virus in human cell monolayers. Derivatives of the related benzoxazole and benzothiazole series are inactive.[87] N-Methyl and N-ethyl isatin β-thiosemicarbazone were shown[88] to directly inactivate the Rous sarcoma virus in vitro. Methisazone was inactive against chickenpox and infectious mononucleosis in a controlled clinical trial.[89] 5-Cyanothiophene-2-aldehyde thiosemicarbazone is reported[90] to be more active than methisazone. The L-enantiomer of cytosine arabinoside was inactive[91] in vitro vs herpes virus. The O_2, O_2' anhydroderivative of cytosine arabinoside did have anti-herpes activity in vitro and in vivo.[91] 1-(3,5-Dihydroxy-4-nitrobenzyl)-morpholine was reported[92] to be active in the mouse against encephalomyocarditis.

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Chapter 13. Antineoplastic Agents

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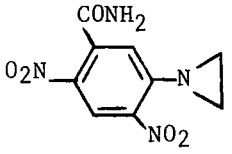
Introduction - An account of a conference on the National Cancer Institute chemotherapy program has been published. Topics included screening, selection of agents for screening, cell kinetics and chemotherapy of cancer, treatment schedule dependency of experimental antileukemic (L1210) drugs, laboratory of toxicology, drug development programs of the National Cancer Institute, clinical trials and combination chemotherapy, and supportive care in cancer therapy.[1]

In addition to the subjects which are presented in this review, other studies beginning to have an impact on the approaches to cancer chemotherapy include molecular orbital calculations and mathematical correlations of lipophilic parameters and partition coefficient measurements of agents of biological importance. Human pituitary growth hormone, prostaglandins, cyclic AMP, RNA-dependent DNA polymerase--their function, relationship, immunological aspects, and influence on other hormones, Poly A:U, and tumor growth--also show promise.

Alkylating Agents - 5-Aziridinyl-2,4-dinitrobenzamide[2] (I) has exceptional activity against the Walker carcinoma. Its antitumor activity is reversed by 4-amino-5-imidazolecarboxamide. Compound 1 is similar to melphalan (L-sarcolysin) in its effects on DNA and RNA synthesis. An azo compound 2, formed in vivo from two molecules of 1, has been proposed as a possible active form.



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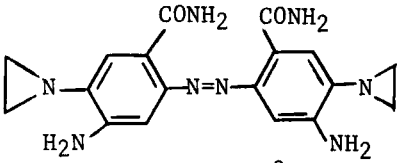

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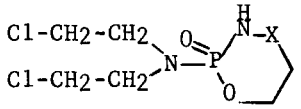
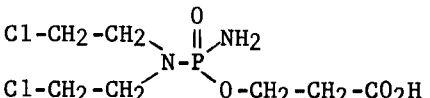
Suppression of cell membrane transport plays a role in the resistance to nitrogen mustard (HN_2) by the Yoshida sarcoma cells[3]. Metabolism of cyclophosphamide (3a, cytoxan) by rat hepatic microsomes has been reported[4]. The active metabolite 4 is formed by a three-step enzymatic reaction.



3a. X = CH_2
b. X = CO



4

4-Ketocyclophosphamide (3b) is an inactive metabolite.[5] Significant synergistic action of potassium iodide on the therapeutic effect of 3 was not confirmed.[6] Busulfan appears to be the treatment of choice for chronic myelocytic leukemia.[7,8]

5-Azacytidine - 5-Azacytidine inhibits the incorporation of tritiated thymidine or deoxyadenosine into DNA to a greater extent than it does tritiated uridine into RNA. The inhibition can be nullified by cytidine, or by uridine, but not by deoxycytidine or deoxyuridine. 5-Azacytidine inhibits the S phase (DNA synthesis) of the cell cycle.[9] The activity of uridine kinase in mouse leukemia cells resistant to 5-azauridine is reduced to ca. 50% of that in the sensitive cells.[10,11] This enzyme is responsible for the initial phosphorylation step of uridine, cytidine, and 5-azacytidine.[9]

Cytosine Arabinoside (1-β-D-arabinofuranosylcytosine, ara-C, cytarabine) - Enzymatic phosphorylation studies confirm that ara-C and its phosphorylated derivatives are deoxycytidine antagonists rather than cytidine antagonists.[12] Inhibition of DNA polymerase activity is considered more significant[13] to antineoplastic properties than either blocking the uptake of thymidine[14] or the inhibition of the conversion of cytidine diphosphate to deoxycytidine diphosphate. Ara-C rapidly kills S phase cells. It affects the passage rate of cells from S to G_2 phase more than from G_1 to S[15].

Ara-C is effective against acute lymphocytic and myeloblastic leukemia,[16] and also in the palliation of advanced epidermoid carcinoma of the head and neck but duration of therapeutic benefit is brief.[17] Unlike 5-FU and MTX, ara-C does not cause mucositis. Recovery of cellular colony-forming unit is much faster after ara-C treatment than with BCNU.[18] A sensitive test for detecting ara-C in plasma has been developed.[19] The 5'-adamantoate of ara-C has been studied as a depot form of ara-C.[20] Many 5'-esters of ara-C possess immunosuppressive and antileukemic activities.[21] The 3-N-oxide derivative retains antileukemic action against leukemia L-1210,[22] but 1-β-L-arabinofuranosylcytosine, the enantiomer of ara-C, has no antineoplastic action.[23,24]

5-Fluorouracil and Related Pyrimidines - 5-Fluorouracil (5-FU) might be more effective in patients with liver metastases when given orally, due to high drug concentrations in the portal system. Oral dosage with 5-FU is safe and effective in metastatic colorectal carcinoma.[25] 5-FU enhances the antitumor effect of irradiation in bronchogenic carcinoma.[26] It supports hepatic tyrosine aminotransferase (TAT) induction by altering in vivo turnover of glucocorticoid-induced TAT.[27] Catabolism of 5-FU is delayed by the presence of 5-cyanouracil.[28] 5-FU

in combination with an anticoagulant (warfarin) increases the antitumor effect.[29]

2'-Deoxy-5-(trifluoromethyl)uridine (F_3TDR) induces remissions in acute leukemias. Doses of F_3TDR causing leukopenia in patients are not accompanied by gastrointestinal toxicity.[30] Prolonged bone marrow depression has hampered the good inhibitory activity of F_3TDR in patients with breast and colon cancer.[31] 5-Bromo-2'-deoxyuridine is incorporated into cellular DNA, but inhibits the transcription of only certain genes into m-RNA.[32]

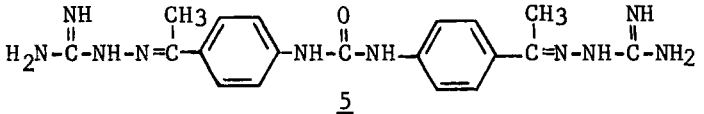
6-Mercaptopurine - 5-Formamide-1-β-ribofuranosylthioimidazole-4-carboxamide 2',3',5'-triformate, is comparable with 6-mercaptopurine (6-MP) for the treatment of leukemias.[33] It is cross-resistant with 6-MP. Conversion of 6-MP ribonucleoside to 6-MeMP ribonucleotide has been shown in L-1210 leukemia cells, adenocarcinoma 755 cells, H. Ep. No. 2 cells and other systems. 6-MeMP ribonucleotide is responsible for the inhibition of purine synthesis produced by 6-MP.[34] Metabolism of 6-MeMP in Ehrlich ascites cells has been studied.[35] Breakdown of the active nucleotide form of the 6-thiopurines by alkaline phosphohydrolase is probably responsible for the resistance of nucleoplastic cells to these agents.[36]

Folic Acid Antagonists - In certain cells folic acid antagonists, such as methotrexate (MTX), homofolic acid or tetrahydrohomofolic acid, inhibit the incorporation of the C-8 carbon into 4-aminoimidazole-5-carboxamide ribotide. Inhibition of DNA synthesis by MTX and related antagonists is not selective, but is balanced by concurrent inhibition of RNA and/or protein synthesis.[37-39] A therapeutic advantage with reduced toxicity is achieved by the administration of high doses of MTX, followed by "rescue" with folinic acid as an antidote.[37,40,41] Dosage schedules and toxicological studies of methasquin, a potent inhibitor of dihydrofolate reductase, were reported.[37,42,43] Unlike MTX, this quinazoline compound is poorly absorbed from the gastrointestinal tract. The N-10 nitrogen atom of folic acid is biologically significant[44] since N-10-deazafolic acid has no antineoplastic activity.[45]

A study of induction of cytotoxicity by MTX has shown that MTX-treated lymphocytes added to allogeneic primary cultures of embryo cells produced a cytotoxic effect; no effect was observed when they were added to syngeneic cells of primary embryo culture. Normal syngeneic lymphocytes when treated with MTX, suppressed tumor cell growth in mice.[46]

MTX inhibits the immune response to insulin. The suppression is reversed by folinic acid.[47] Vitamin B_{12} promotes DNA-thymidine synthesis in C_3H mouse cells by raising the level of folate coenzymes.[48]

[N,N'-(4,4'-Diacetyl)diphenyl]urea Bisguanylhydrazone (DDUG) - DDUG (5) inhibits a number of leukemias and some mammary tumors in mice. Resistance to DDUG is associated with a reduced uptake of the compound, but the intracellular distribution and drug binding was unchanged. Strains resistant to DDUG are cross-resistant to 2-chloro-4',4"-bis(2-imidazolin-2yl)terephthalanilide, but remain sensitive to methylglyoxal bis(guanylhydrazone) and vincristine. The sites of inhibitory action of all four agents are different.[49]



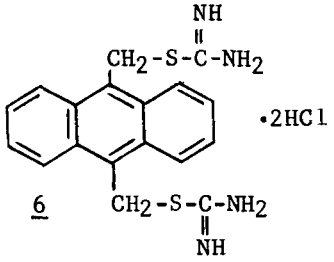
5

Hydroxyurea - The cytotoxicity of hydroxyurea was compared in normal and in rapidly proliferating epidermis;[50] deoxyribonucleoside incorporation was investigated in alkylated, non-dividing human lymphocytes.[51] The absorption, distribution, and excretion of hydroxyurea in patients was reported.[52] The cytotoxicity of hydroxyurea is due to inhibition of ribonucleotide reductase in DNA synthesis.

Hydroxyurea inhibits skin carcinogenesis;[53] activity against non-resectable cancer of the lung is comparable with that of cyclophosphamide.[54] It is an effective agent in epidermoid carcinoma of the head.[55] However, clinical trials against malignant melanoma[56] and acute myelocytic leukemia[57] are discouraging.

Nitrosoureas - 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) are extremely lipid soluble and readily cross the blood-brain barrier.[58] CCNU is more efficacious than BCNU against implanted gliomas and leukemia L-1210. Clinically, BCNU and CCNU are effective in patients with bronchogenic carcinoma, malignant lymphoma and acute leukemia in children.[59] Their most important use is for the treatment of patients with malignant brain tumors.[59-62] Because of their lipid solubility and relative nonionic character, nitrosoureas are distributed widely in plasma, cerebral spinal fluid, brain, muscle and other tissues. They are readily metabolized; only a small amount of parent material can be isolated from tissues together with large amounts of metabolites.[62,63] The antineoplastic activity of CCNU may be due to modification of cellular proteins and to alkylation of nucleic acids.[63] Nitrosoureas act on marrow stem cells, cause a double fall in the WBC count, and induce a more prolonged reduction in colony-forming units than does cytosine arabinoside.[18] Nitrosoureas often cause delayed bone marrow toxicity, transient hematocrit depression and hematopoietic suppression, which may be related to the drug-induced alterations in the cell cycle.[64] This effect may be relieved by a change in dosage regimen.[62] Structural modifications of N-nitrosoureas are reported.[65]

"Pseudourea" - 2,2'-(9,10-Anthrylenedimethylene)bis-(2-thiopseudourea) dihydrochloride (6), has shown activity against mouse leukemia L-1210. It complexes with DNA and inhibits both DNA synthesis and DNA-dependent RNA polymerase activity.[66] Initial clinical studies revealed undesired phototoxicity in most patients with metastatic cancer.[67]

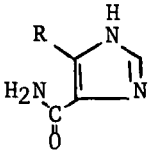


6

5-(3,3-Disubstituted 1-triazeno)imidazole-4-carboxamide (7 DIC) - DIC is used for treating disseminated malignant melanoma;[68,69] it is reported to have activity against carcinoma of the lung[70,71] and primary brain tumors.[71] However, it is of no use in malignant melanoma in Uganda.[72] Bone marrow depression, vomiting, diarrhea, oral ulceration, hepatocellular damage, CNS effects, fever, and a delayed flu-like syndrome are reported side effects.

DIC is transformed to 9 via 5-diazoimidazole-4-carboxamide (8).[73] DIC undergoes stepwise demethylation to 5-aminoimidazole-4-carboxamide (AIC).[74,75] The monomethylated intermediate (10)[75] or diazomethane[76] may be the active metabolites of DIC. The imidazole-4-carboxamide 11 (BIC) is transformed into an ionic product 12 [77] in vivo, which may explain the differences in antileukemic action and resistance development of DIC and BIC.[75] Cytotoxicity study[78] and preliminary clinical trials[76] of BIC are reported. The action of 5-diazoimidazole-4-carboxamide (8) was studied in rabbit platelets; the sulfhydryl group and pyrophosphate structures may be involved in the mechanism of release of 5-hydroxytryptamine by 8.[79]

Two isomeric compounds of DIC were synthesized. 5-(3,3-Dimethyl-1-triazeno)pyrazole-4-carboxamide (13) is stable toward light and heat; it has good activity against leukemia L-1210 and less toxicity than 7.[80] The isomeric 5-carboxamide 14 lacks antileukemic activity.[80]

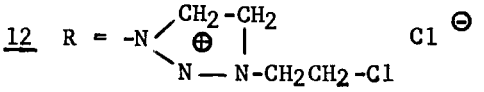


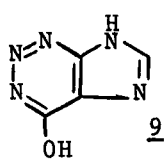
7 R = N=N-N(CH$_3$)$_2$

8 R = (N$_2$)$^+$

10 R = N=N-NH(CH$_3$)

11 R = N=N-N(CH$_2$CH$_2$Cl$_2$)

12 R = 



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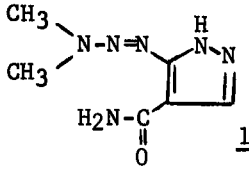

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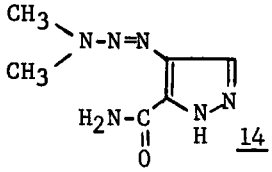


14

Steroids - Large doses of estrogen inhibit prolactin stimulation of mammary tumor growth as demonstrated by the effect of estradiol benzoate to DMBA-induced mammary adenocarcinomas in female rats.[81] Estrogen may be involved in transport of RNA from nucleus to cytoplasm.[82] Specific binding of estradiol within the nucleus is confined to hormone-dependent tumors.[83]

6α-Methylpregn-4-ene-3,11,20-trione produces favorable responses against malignant melanoma, carcinoma of the breast and prostate with only mild toxicity when given orally.[84] Hydroxyprogesterone caproate gave excellent responses in adenocarcinoma of the uterine corpus.[85] Preliminary clinical studies with phenesterin in patients with advanced breast cancer were disappointing.[86] A spectrophotometric analysis for diethylstilbestrol diphosphate has been developed.[87] Androsterone deficiency is associated with poor prognosis of lung cancer.[88]

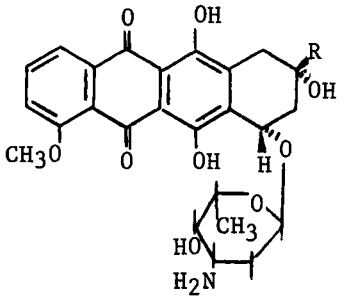
1-(o-Chlorophenyl)-1-(p-chlorophenyl)-2,2-dichloroethane (o,p'-DDD) - o,p'-DDD produces favorable clinical responses of adrenocortical carcinoma in man is effective in an adrenal preparation in vitro but not in the adrenal slice. o,p'-DDD does not affect base-line steroid production when ACTH-induced steroidogenesis is completely blocked, which indicates that o,p'-DDD interferes with the mechanism by which ACTH stimulates steroidogenesis.[89]

Actinomycin D - Kinetic studies with actinomycin D (act-D) in transplanted leukemic mice showed that a single dose provided cytotoxic concentrations for up to 24 hours.[90] The characteristics of act-D resistance in L5178Y cells show that alterations in membrane composition and conformation in the drug-resistant subline accounts for the observed changes in the permeability of this drug.[91] Act-D enhances the toxicity of morphine by increasing brain permeability.[92]

Exposure of leukemia P388 to act-D causes respiratory depression, a decrease in malic and lactic dehydrogenase and an increase in glucose-6-phosphate dehydrogenase. These effects are similar to those caused by nitrogen mustard.[93] Act-D stimulates isoperoxidase activity at low concentration and represses it at higher concentration.[94] It inhibits the activity of DNA polymerase.[95]

Vitamin A acid causes mucous metaplasia in the skin tumor keratoacanthoma.[96] Mucus was not observed after topical application of act-D to the skin tumor prior to the usage of Vitamin A acid.[97]

Adriamycin and Daunorubicin - The structure and absolute stereochemistry of daunorubicin (15a daunomycin, rubidomycin) were determined.[98]



15a. R = $CO-CH_3$
b. R = $CHOH-CH_3$
c. R = $CO-CH_2OH$

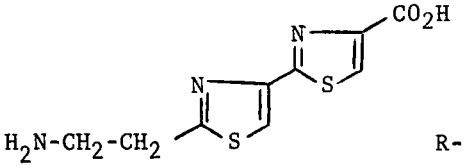
Clinical trials of 15a in children with acute lymphoblastic leukemia were compared and reviewed.[99] This antibiotic has some value in children with advanced disseminated neuroblastoma,[100] but may ultimately find its use in combination chemotherapy.[101,102] Daunorubicin is rapidly metabolized in rats to daunorubicinol (15b), and the aglycones of 15a and 15b.[103] Compound 15b was isolated from human urine and enzymatic reactions.[104] Daunorubicin (15a) and adriamycin (15c) complex strongly to DNA, inhibit mitotic activity, DNA synthesis[105,106] and proliferation of HeLa cells. Daunorubicinol is only slightly active in inhibiting DNA synthesis and cellular proliferation.[106] When the amino sugar of these compounds is masked or exchanged for α-D-glucosamine, the resulting derivatives lack biological activity. The ability of these compounds to bind DNA is associated with the structure of the amino sugar.[105] This observation supports the antileukemic triangulation pharmacophore hypothesis.[44]

The pharmacokinetics, pharmacology, chemotherapeutic effects, toxicity, chromosome aberations and mutagenic potency of adriamycin were reported.[105-109] Adriamycin is useful for acute lymphoblastic and chronic myelogenous leukemias, transitional cell carcinoma, liposarcoma, squamous cell carcinoma, and adenocarcinoma of the breast. It is less toxic than daunorubicin. Resistance to daunorubicin is due to changes in the cellular membrane.[110] Both antibiotics possess immunosuppressive properties.[111]

Bleomycins - Complete structures of the bleomycins have not been elucidated. Bleomycin A_2 is active against the ascites type of Ehrlich carcinoma and sarcoma 180 in mice, and is clinically useful in the treatment of human epidermoid cancer, squamous cell carcinoma of the head and neck, lymphosarcoma, Hodgkin's disease, mycosis funoides, Kaposi's sarcoma, carcinoma of the thyroid, and brain tumors.[112-114] It exhibits very

low renal toxicity and rarely causes leukopenia, thrombocytopenia or hematopoiesis in patients. Bleomycin A_2 frequently causes pulmonary toxicity. It inhibits DNA synthesis in E. coli, Ehrlich carcinoma and HeLa cells, inhibits ATP-dependent DNA ligase activity, causes DNA strand scission in vivo and in vitro, and decreases the melting temperature of DNA in the presence of hydrogen peroxide or sulfhydryl compounds.[115,116] It prevents cells from entering visible mitosis.[117] The activity of DNA polymerase from rat ascites hepatoma AH-130 cells is reduced by bleomycin but in vitro addition of this antibiotic to the DNA polymerase assay system does not produce noticeable inhibition.[118] An increase in the number of chromosomal aberrations was observed in cancer patients following administration of the antibiotic.[119]

The sulfur-containing chromophore (16), which is the "core" of all bleomycins, was revealed through x-ray analysis and confirmed by synthesis.[120] Partial structure of bleomycins A_2, A_2', A_5, A_6, B_1 and B_2 are represented by 17. In bleomycin A_2, X is 3-aminopropyldimethylsulfonium halide; in other bleomycins X is a straight chain polyamine.



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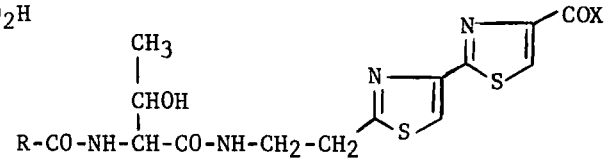

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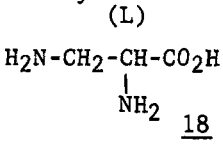
Bleomycin A_2 (M.W. ca 1400) contains two sugars (l-glucose and 3-O-carbamoyl-D-mannose) and the peptide and amino acids 18-20.[121]



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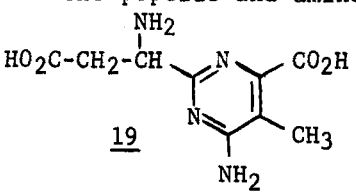

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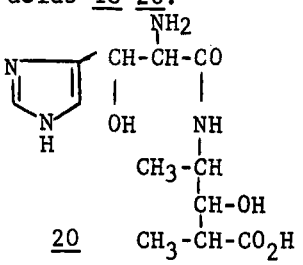

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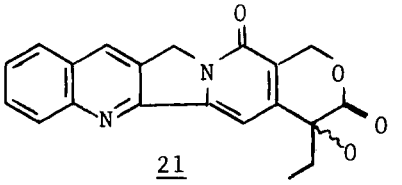
Mitomycin C - Human sarcoma cells exposed to mitomycin C stimulated DNA synthesis in autochthonous lymphocytes. Preexposure of the tumor cells to autochthonous serum abrogated lymphocyte stimulation.[122] The use of mitomycin C in combination with cycle-phase specific drugs that may block cell repair, or with agents having a different mode of action has been suggested,[123] but preliminary combinations with phenylalanine mustard, and vincristine in the treatment of osteogenic sarcoma was without much effect.[124]

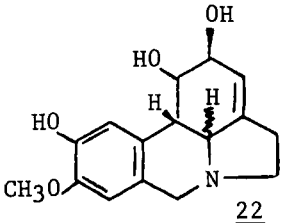
Camptothecin - An alkaloid from the tree Camptotheca acuminata, camptothecin (21) exhibits antineoplastic activity against the mouse leukemias, the plasma cell tumor YPC-1, Walker 256 rat carcinosarcoma and has cytotoxic activity against several cell lines.[125-127] Total synthesis[128,129] and preliminary structure-activity relationship studies[130] are reported. Camptothecin is not cross-resistant to leukemias made refractory to the antifols, BCNU, cytosine arabinoside, 6-mercaptopurine, L-asparaginase, or vincristine.[127] The primary effect of this alkaloid is on DNA synthesis. It also causes a G_2 "lesion" which prevents subsequent mitosis. The major effect of 21 is to affect cells in S phase and the capacity of these cells to go through G_o phase.[127] Inhibition of DNA synthesis in L-1210 cells by camptothecin is initially reversible, but becomes irreversible when exposure to the drug is prolonged.[131] Camptothecin resistant L-1210 subline is not characterized by a drug permeability barrier nor by altered levels of cell surface glycoprotein, which differ from characteristics of actinomycin D-resistance cell lines.[131]



21

Camptothecin inhibits ribosomal RNA synthesis without affecting transfer RNA or protein synthesis;[132,133] the RNA synthesis inhibition is rapidly reversible upon removal of the drug.[133] Initial claims of clinical activity of camptothecin in gastrointestinal cancer[134,135] have not yet been confirmed.

Among other antineoplastic agents of plant origin, the narcissus alkaloid pseudolycorine (22) is active against Rauscher leukemia.[136] Taxol, from Taxus brevifolia, is active against a number of experimental neoplastic systems; its structure (23) was reported.[137] The sesquiterpene lactones[138,139] and bufadienolides[140] are cytotoxic.



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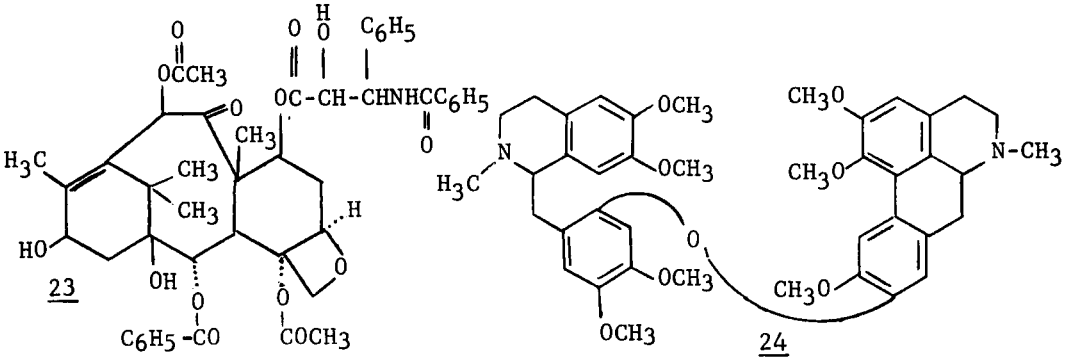


23 24

The factor responsible for cytotoxicity of the sesquiterpene lactones is the $O=\dot{C}-\dot{C}=CH_2$ moiety.[138] Total synthesis of the tumor-inhibitory aporphine alkaloids, thalicarpine (24) and hernandaline was accomplished.[141]

Emetine - As an inhibitor of protein synthesis at the transcription level, emetine is also active against leukemias L-1210 and P-388, B16 melanomas, Ehrlich ascites carcinoma and Yoshida sarcoma. Pharmacologic studies showed that it effects a sympathetic blockade, antagonizes hyaluronidase, inhibits oxydative N-demethylation of aminopyrine and N-ethylmorphine as well as the S-demethylation of 6-MP riboside in vitro.[142,143]

Preliminary clinical trials indicated that emetine reduced lung tumor size and purulent bloody vaginal discharge; severe muscle weakness is the most important dose-limiting toxic effect. Emetine is not myelosuppressive and may be useful in patients with poor marrow reserve.[144]

Cardiotoxic effects of this alkaloid and its 2-dehydro analog were noted in rabbit heart. The observed cardiopathy compares with those not accompanied by vascular symptoms but associated with necroses due to electrolyte and steroid disturbance.[145] N-Substituted ℓ-emetine derivatives have also been found to inhibit growth of Ehrlich ascites carcinoma.[146]

Vitamin A Acid - Vitamin A acid is capable of inducing regressions in a benign as well as malignant epithelial tumor. It has a therapeutic effect on established skin papillomas and skin carcinomas induced by 7,12-dimethybenz[a]anthracene and croton oil.[147] Initial clinical trial has established the effect of topically applied vitamin A acid on basal cell carcinomas on the skin in man.[148]

L-Asparaginase - Two L-glutamine antagonists (6-diazo-5-oxo-L-norleucine and azaserine) produce additive antileukemic effects with L-asparaginase in mouse leukemia L5178Y.[149] Rapid development of L-asparaginase resistance was observed.[149] L-Asparaginase from different sources possesses varied tumor inhibitory properties. The enzyme from Serratia marcescens is more inhibitive against 6C3HED lymphoma than that from E. coli.[150] L-Asparaginase has a weak activity in adult acute myelocytic leukemia; it produces about 50% remissions in adults with acute lymphocytic leukemia[151] and has some activity in children with advanced leukemia.[152,153] It will have to be combined with other drugs if it is to significantly affect survival of leukemia patients.[154] Acrolein in tobacco smoke[155] and hormonal antibodies[156] reduce the therapeutic activity of L-asparaginase.

L-Asparaginase is a potent immunosuppressive agent.[157-159] This effect was not decreased by heat treatment or simultaneous administration of L-asparagine.[160] L-Asparaginase from E. coli has a tetrameric structure with identical units, each possessing a single catalytic site.[161,162] Extensive cross-linkage and alterations of substrate specificity can occur when the enzyme is treated with tetranitromethane.[163] The native enzyme has 10% α-helices and 45% β-structure; the rest are of the unordered types. Lyophilization increases the content of β-structure.[164] Production by tumor cells and the effect of asparagine analogs were studied.[165] Its action on mitotic activity during induced hepatocarcinogenesis was reported.[166]

t-RNA Methyltransferase - Abnormally high levels of methyltransferase enzymes and methyltransferase activity were found in neoplastic tissues including virally induced, chemically induced, and spontaneous tumors.[167-171] The t-RNAs of many tumors contain highly elevated amounts of N-methylated, C-methylated, and the 2'-O-methylated nucleosides.[168] t-RNA from solid human tumors with t-RNAs from normal tissues was compared chromatographically.[172] t-RNAs are associated with the regulation of protein synthesis at the translation level and since alkylating carcinogens were found to alkylate t-RNA in vivo, the aberrancy of methyltransferase could be involved in the initiation of tumor induction and neoplasia.[168] Inhibitors of methyltransferase are postulated to be of potential value in cancer chemotherapy.[168]

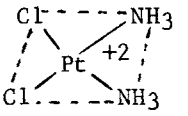
Ricin - A phytotoxic protein from Ricinus communis is inhibitory towards protein synthesis in experimental tumor cells. It has a moderate inhibitory effect on DNA synthesis without affecting RNA synthesis. Its mode of action is not known, but is not due to impairment of glucose metabolism nor amino acid uptake in tumor cells.[173]

Gallium Salts - With a special affinity for malignant tumors, gallium salts are used as diagnostic tools for detection of neoplastic bone metabolism and allied neoplastic diseases.[174,175] Gallium bromide, chloride, citrate, iodide, and nitrate as well as ammonium gallium chloride have antitumor activity against Walker carcinosarcoma 256 and P-1798 lymphosarcoma. Gallium 5-fluoroörotate is active against leukemia L-1210 in mice.

Autoradiographic studies of gallium-67 salts in rodent tissue show more than 70% of the ^{67}Ga activity was localized in the cytoplasm. High concentrations of ^{67}Ga were observed in the epithelium of renal convoluted tubules.[176] Four Group III-A elements (aluminum, gallium, indium, and thallium) had some antitumor activity but only Ga^{+3} and, to a lesser extent, In^{+3} inhibited tumor growth when the tumor was innoculated by a

route different from that of the salts. The decreasing order of toxicity was $In(NO_3)_3 \gtrsim TlCl_3 > Ga(NO_3)_3 > Al(NO_3)_3$. Some gallium salts may be useful for treatment of solid tumors in man.[177,178]

Platinum Complexes - cis-Diamminedichloroplatinum (25), exhibited potent antitumor activity against sarcoma 180, Ehrlich ascites carcinoma, leukemia L-1210, Dunning ascites leukemia, Walker 256 carcinoma, DMBA-induced mammary carcinoma, and virus-induced reticulum cell sarcoma in experimental animals.[179,180]



25

These platinum complexes cause a consistent inhibition of DNA synthesis in vivo.[181] At very low concentrations, 25 selectively inhibited the incorporation of thymidine-CH_3-^3H, uridine-5-^3H, L-leucine-^{14}C and L-leucine-^3H into mammalian cells.[181,182] It may undergo sequential transformations with loss of chloride, and the resultant platinum species may act bifunctionally to crosslink adjacent nuclephilic centers of DNA through covalent binding.[183]

The acute toxic and pathogenic effects of 25 in rats include pronounced effects on many of the rapidly proliferating normal tissues (i.e., in the intestines, the marrow, and the renal tubules).[184,185] Hematologic studies show a decrease in circulating reticulocytes and lymphocytes after administration of 25, and histologic studies showed striking changes in thymus and spleen.[186] Deafness was noted in preclinic trials.

An aryl congener of 25, cis-dichlorodipyridineplatinum, possessed actions similar to those of the inorganic species, albeit less potent.[187]

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Chapter 14. Antiparasitic Agents

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Introduction - The recent literature contains many citations to parasite-related diseases and problems pertaining thereto. In a recent paper[1] several areas requiring intensified research and the development of new or improved drugs were identified. In the past year, a single periodical has carried papers on amebicides,[2] anthelmintics,[3] malaria,[4,5] amebic and bacterial dysentery,[6] intestinal helminths and filariasis,[7] trypanosomiasis and leishmaniasis,[8] schistosomiasis,[9] and immunodiagnosis of parasitic diseases.[10] Considerable information relating to current treatment of these afflictions may be found in these papers as well as in a report called "Drugs for Parasitic Infections".[11]

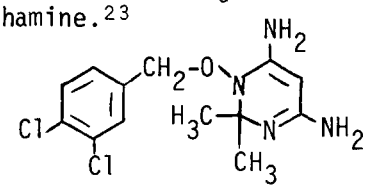
The proceedings of two recent symposia have been published, i.e., on methodology for the development, selection and testing of anticoccidial drugs[12] and on the development of drug resistance in malaria.[13]

Evidence is accumulating that natural or acquired immunity to pathologic parasite infections is abolished by immunosuppression, particularly by betamethasone, a corticoid.[14-16] Consequently latent parasitic infections may flare up in patients being treated with cortisone or its derivatives.[17,18]

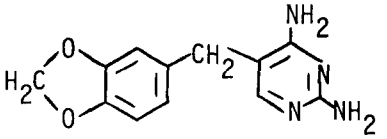
Antimalarials

General - Application of an automated method of mass drug testing to antimalarial screening has been proposed.[19] It has been suggested that CMLR-1066, a commercial culture medium, is useful for testing susceptibility to antimalarial drugs.[20]

Identical dihydrofolate reductase enzymes, isolated from P. berghei and P. knowlesi, have been found to be sensitive to pyrimethamine. There is no evidence that inhibition of the enzymes leads to inhibition of DNA synthesis.[21,22] Other dihydrofolic acid reductase inhibitors, cycloguanil, W.R. 38839 (I), trimethoprim, methotrexate and W.R. 40070 (II), the latter three at higher concentrations, acted in a manner similar to pyrimethamine.[23]



I



II

Adenosine, tritiated in the 8-position, is incorporated into the RNA and, to a lesser extent, into the DNA of P. berghei. Indications are that adenosine monophosphate is degraded to adenosine by erythrocytes before it

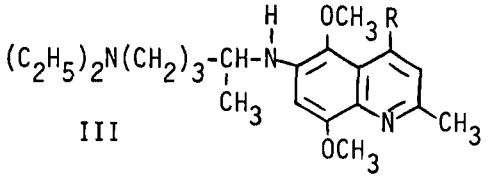
can enter the parasite.[24,25]

Labeling experiments show that blood cells infected with P. knowlesi and P. falciparum synthesize coenzyme Q (ubiquinone) from p-hydroxybenzoic acid and that coenzyme Q_8 is apparently the dominant coenzyme Q for P. knowlesi, P. lophurae and P. cynomolgi.[26] Consequently the synthesis of potential reversible inhibitors of the biosynthesis of function of coenzyme Q_8 has been undertaken.[27]

An attempt has been made to quantitate what appear to be qualitative structure-activity relationships for chloroquine analogs.[28]

Resistance to chloroquine and other antiplasmodials - The development of resistance has caused grave concern.[13] Chloroquine halts degradation of hemoglobin by the malarial parasites which then succumb to amino acid starvation. Experiments indicate[29] that chloroquine resistance develops by a switch in the trophozoite stage to the normally lacking citric acid cycle from which amino acids are produced. Alternatives to overcome drug resistance were reviewed in 1968[30] and additional suggestions have appeared in the most recent literature.[31-34]

The search for new antimalarials - A new cinchona alkaloid synthesis paved the way to various quinine analogs[35] of which the 6'-desmethoxy-7'-chlorodihydroquinines were found to be 4 times as potent as natural quinine against P. berghei in mice. The natural and unnatural antipodes and racemic forms of the alkaloids showed no appreciable differences in activity.[36] Increased activity was found among some 2'-alkyl- and aryl-quinine derivatives the most active of which are useless, however, because of phototoxicity.[37] Compounds IIIa and IIIb are new quinoline antimalarials comparable to, or superior in activity to chloroquine and primaquine against P. vinckei (mice) and P. cathemerium (canaries). Significant cross resistance to chloroquine was found in P. berghei with IIIb but none with IIIa.[38] The weak antimalarial menoctone[30] (naphthoquinone analog of IVa or IVc) exerted some inhibition of coenzyme Q function. Compounds

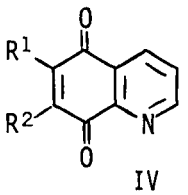


III

a: R=CH_3

b: R=OCH_3

IVa-d were better coenzyme Q inhibitors and compared favorably with menoctone as antiplasmodials. Hydrogenation of the heterocyclic ring destroyed the activity.[26] Compound (Va) and analogs,notably Vb, structur-



IV

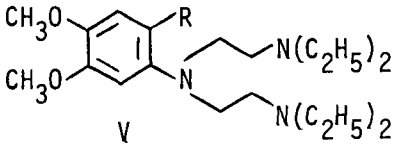
a: R^1=cyclohexyloctyl, R^2=OH

b: R^1=n-pentadecyl, R^2=OH

c: R^1=OH, R^2=cyclohexyloctyl

d: R^1=OH, R^2=n-pentadecyl

ally reminiscent of coenzyme Q, have significant antiplasmodial activity. Structural variations have little effect on activity.[39] The best of re-

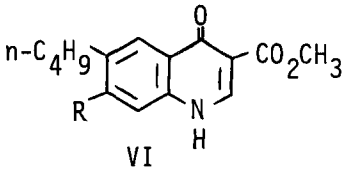


V

a: R=H

b: R=Br

cently prepared side chain analogs of chloroquine are comparable in activity to chloroquine.[40,41] Analogs of the coccidiostat benzoquate (VIa) have been recognized as antimalarials; ICI 56780 (VIb) and ICI 60128 (VIc) supress P. berghei asexual erythrocytic infections in mice at doses of 1 mg/kg s.c. and both are potentiated by sulfadoxine.[42] ICI 56780 acts prophylactically against P. berghei in mice and P. cynomolgi in rhesus monkeys. B-663, an antileprosy and antitubercular drug,[43] has been



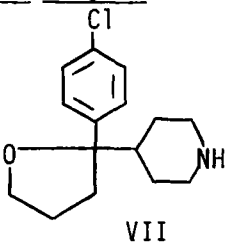
VI

a: R=$C_6H_5CH_2O$-

b: R=$C_6H_5O(CH_2)_2O$-

c: R=n-$C_8H_{17}O$

found to be active in chloroquine resistant strains of Plasmodium.[44] Novel structure BA-41799 (VII) is active against P. chabaudi[45] and strains of P. berghei resistant to chloroquine, mepacrine, primaquine, cycloguanil and diaminodiphenylsulfone (DDS).[46] Sulfamonomethoxine monohydrate (DJ-1550) is reported to be particularly active against P. falciparum and P. vivax.[47] New antifolates and antifolate combinations have become increasingly important as antiplasmodials[23,32,34,48,49] and it is along these lines that future progress can be expected.

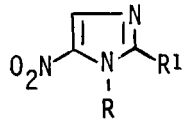


VII

A caution was recently sounded with respect to the use of DDS which is partly metabolized to a toxic, methemoglobin-producing hydroxylamine derivative.[50]

Nitroimidazoles

The use of nitroimidazoles for the treatment of protozoan and, to some extent, metazoan infections is becoming increasingly more important. Furthermore a given member of this class of compounds is frequently effective against a variety of parasites. The 5-nitroimidazoles have generally proven to be superior to the 4- and 2-nitroimidazoles[51] as antiparasitic agents but the latter seem to have better antibacterial properties.



VIII

a: $R=CH_3$, $R^1=CH_3$

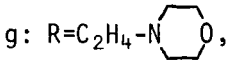
b: $R=CH_2CH_2OH$, $R^1=CH_3$

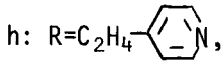
c: $R=CH_3$, $R^1=CH_2OCONH_2$

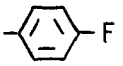
d: $R=CH_3$, $R^1=CH(CH_3)_2$

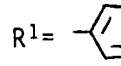
e: $R=CH_2CHOHCH_2Cl$, $R^1=CH_3$

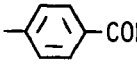
f: $R=(CH_2)_2SO_2C_2H_5$, $R^1=CH_3$

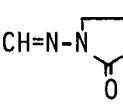
g: 

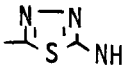
h: 

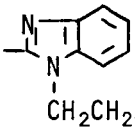
i: $R=CH_3$, $R^1=$ 

j: $R=CH_2CH_2OH$, $R^1=$ 

k: $R=CH_3$, $R^1=$ 

l: $R=CH_3$, $R^1=$ 

m: $R=CH_3$, $R^1=$ 

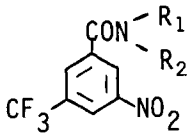
n: $R=CH_3$, $R^1=$ 

Dimetridazole (VIIIa) continues to be used in veterinary medicine against trichomonas in cows[52] and Histomonas meleagridis infections in fowl.[53] Metronidazole (VIIIb), the standard drug for Trichomonas vaginalis and T. foetus infections, has gained some prominence as an intestinal and hepatic amebicide,[54-56] but more recently attention has been called to the potential development of trichomonal resistance.[57] Ronidazole (VIIIc)[58] and ipronidazole (VIIId)[59-61] are potent histomonastats. The latter substance is not only more active against H. meleagridis than closely related nitroimidazoles including dimetridazole,[62] but it also has superior antitrichomonal properties.[63] Compound VIIIe (Ro 7-0207) has been found to be highly active against Entamaeba histolytica, intestinal in rats and hepatic in hamsters, and effective against pinworms.[64] Tinidazole (VIIIf),[65] compared with metronidazole, has been found to be 4-16 times as active against T. vaginalis and T. foetus and about equal against E. histolytica. It was also found to be active against Eimeria tenella and H. meleagridis but ineffective toward Trypanosoma brucei, T. congolense, T. cruzi and P. berghei.[66] Nitrimidazole (VIIIg) has been tried clinically as a trichomonastat[67,68] but it was reported to be inferior to metronidizole.[69] Panidazole (VIIIh), when tested clinically as an amebicide, was found to be effective at doses of 100 mg, 2 times daily for 4 days.[70] MF-nitroimidazole (VIIIi) was reported to be particularly effective in amebiasis, flunidazole (MK-915) (VIIIj) against T. vaginalis and T. foetus and MCA-nitroimidazole (VIIIk) against T. brucei in mice.[71] Clinical efficacy for VIIIi was demonstrated.[72] Compound VIIIl was reported to be equivalent to metronidazole against trichomonas.[73] CL-64855 (hydrochloride, CL-75805) (VIIIm), previously cited for its antibacterial activity[74] has now been reported to be 7-8 times as potent as

metronidazole against trichomonas.[75] It has been found to be an effective intestinal and hepatic amebicide[76] as well as being active against T. equiperdum in mice at doses of 20-40 mg/kg and against Leishmania donovani in mice when fed at levels of 0.04% in feed for 7 days.[77] It is, however, 4 times as toxic as metronidazole.[75] Compound VIIIn has been reported to be an effective trichomonacide.[78] Clinical efficacy as an amebicide has been reported for BT-985, a nitroimidazole of undisclosed structure; amebic hepatitis[79] and a case of lupus erythematosus also responded.[80] A second nitroimidazole of undisclosed structure, MK-910, was also reported to be effective,[81] but inferior[82] to metronidazole, as an amebicide.

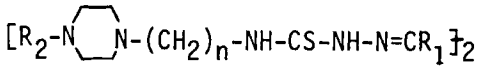
Coccidiostats

A method of evaluating coccidiostats under simulated field conditions has been reported.[83] In addition to the antibiotic monensin,[84,85] the related dianemycin[86] and antibiotic X-206[87] are reported to be effective in the control of coccidiosis. The α,α,α-trifluorotoluamides IX are reported to be active at doses of 0.0065% in feed[88] and the piperazine-substituted dithiosemicarbazones X are claimed in the patent literature[89]



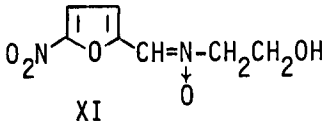
IX

R_1, R_2=H, CH_3, C_2H_5



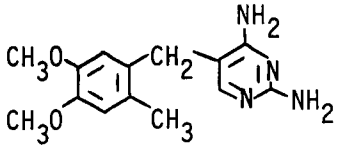
X

to be coccidiostats. Nitrone XI and analogs are reported to be active against E. tenella and also H. meleagridis.[90] Rofenaid® [Ro 5-0013, sul-

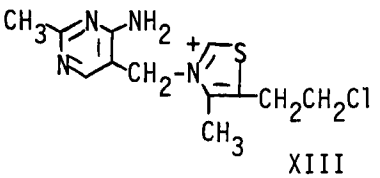


XI

fadimethoxine + ormetoprim (XII)[91] in a ratio of 5:3] is completely effective against a variety of Eimeria strains at the 0.02% level in feed.[92] Another treatment for coccidiosis is the use at 0.0125% in feed of beclotiamine (XIII), a derivative of thiamine. This substance has little or no antithiamine activity in chickens or rats.[93]



XII



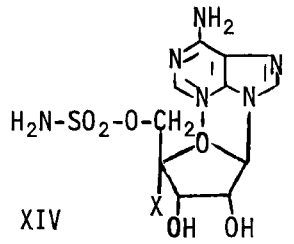
XIII

Histomonastats

A comparative study of several established histomonastats including 4-nitrophenylarsonic acid, p-ureidobenzenearsonic acid, 3,5-dinitrosalicylic acid-5-nitrofurfurylidene hydrazide[94] and dimetridazole was published.[53] The 5-nitroimidazoles ronidazole[58] and ipronidazole[59-61] are superior new histomonastats.

Antitrypanosomal Agents

The need for new antitrypanosomal agents was pointed out in a joint report of the FAO and WHO expert committee on African trypanosomiasis.[95] It has already been noted that some of the nitroimidazoles are active in this area, i.e. CL-64855 (VIIIm) against *T. equiperdum* in mice and *L. donovani* in mice[68] and MCA-nitroimidazole (VIIIk) against *T. brucei* in mice.[71] The antimalarials 2,4-diamino-6-(3,4-dichlorobenzylamino)quinazoline and its nitrosamine derivative CI-679 were found to supress, but not eradicate, *T. cruzi* in mice at doses of 60 mg/kg/day and 46 mg/kg/day for 6 to 8 days.[96] N-diethylaminoethyl-5,6-dihydrocarbazole, the best of a series, has been found to be useful as an additive to donor blood to prevent the spread of Chagas-Mazza disease.[97] 5'-O-Sulfamoyladenosine (XIVa), an analog of the antibiotic XIVb is reported to be 100% curative at single i.p. doses of 2 mg/kg or at 0.1 mg/kg/day i.p. for 3 days.[98] Trypanomycin (A-10091/A), a new pigment antibiotic is reported to be active against trypanosomes as well as several bacteria.[99]



XIV

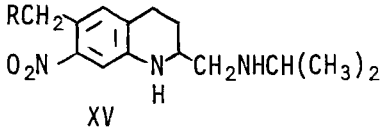
a: X=H

b: X=F

Antischistosomal Agents

General - Schistosomiasis and its treatment have recently been reviewed.[100-102] Moreover, the development of screening tests for potential drugs and assays for the disease have received considerable attention. One proposed *in vitro* screen for schistosomacides uses the cercarial or schistosomular form of *S. mansoni* and a fixed concentration of the test substances.[103] An assay for schistosomiasis uses a fraction of *S. mansoni* antigen labeled with radioactive iodine.[104] A group of 420 compounds, among them being 27 chemical enzyme inhibitors, 4 antischistosomal drugs and 2 specific inhibitors for trypsin and chymotrypsin, were tested for schistosome hemoglobin protease inhibition[105] on the theory that ingested host hemoglobin is required for nutrition of the parasite.[106] A comparison of the established schistosomacides mirasan, lucanthone, niridazole and hycanthone has been published.[107] Hycanthone is still widely used in single i.m. doses of 3 mg despite the unpleasant side effects, mainly emesis, anorexia and abdominal pain.[108] It has been reported that thiosinamine (allylthiourea) blocks normal egg formation in *S. mansoni* by inhibiting the activity of polyphenoloxidase.[109]

New Chemotherapeutic Agents - A single 50 mg/kg p.o. dose of MK-3883 (XVa)[110-111] cures infected monkeys. Single 5-7.5 mg doses of a metabolite (XVb) are active. Compounds XVI a,b,c[112] of a group of naphtho-

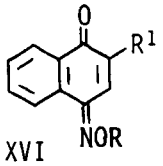


XV

a: R=H

b: R=OH

quinone mono-oximes, and XVII,[113] of a group of nitrosophenols, were found to be particularly active. A recent patent[114] claims antischistosomal ac-

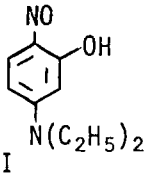


XVI

a: R=CH_3, R^1=H

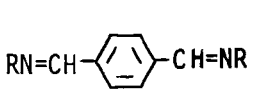
b: R=$COCH_2Cl$, R^1=H

c: R=H, R^1=CH_2-N⬡

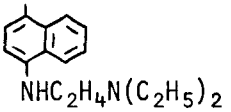


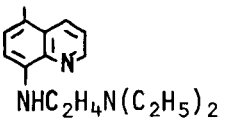
XVII

tivity for the 1,4-diaminonaphthalene derivative XVIIIa and its quinoline analog XVIIIb. Antimonyl-dimethylcysteino-tartrate (NAP) gave 94% cures

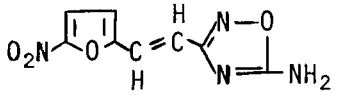


XVIII

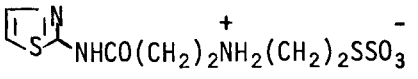
a: R= 

b: R= 

in humans infected with S. mansoni with 5 daily doses of 8% NAP solution, equivalent to a total dose of 2 g or 290 mg of antimony per person.[115] SQ-18506 (XIX)[116] is very active in mice, hamsters,[117] and rhesus monkeys[118] infected with S. mansoni. Attention has been directed to the conformational analogy between SQ-18506 and niridazole.[119] S-2-([2-(2-Thia-



XIX



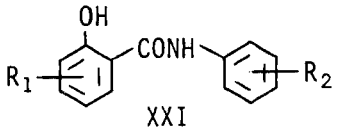
XX

zoylcarbamoyl)ethyl]amino)ethyl acid thiosulfate (XX), its thiol hydrochloride and disulfide have been reported to be highly effective against S. mansoni in mice and monkeys.[120] 2-(2-Methyl-4-amino-5-pyrimidinylcarbamide)-5-nitrothiazole, an antimetabolite of Vitamin B_1, is a potent schistosomacide the activity of which is destroyed, however, by simultaneous administration of high doses of vitamin B_1.[121]

Anthelmintics

Three weeks after inoculation of rats with metacercariae, glutamic-oxaloacetic transaminase appears to be greatly elevated. Since treatment with various fasciolacidal agents caused normalization of the enzyme

levels within one week, it was suggested that this observation be used as a means of evaluating potential drugs for liver fluke infestations. The following substances were found to be active at the indicated levels (mg/kg): hexachlorophene (50), bithionol (300), menichlopholan (5), 3,4',5-tribromosalicylanilide (100), 3'-chloro-4'-(p-chlorophenoxy)-3,5-dibromosalicylanilide (300), 5-bromo-3'-chloro-4'-(p-chlorophenoxy)-3-nitrosalicylanilide (50), oxyclozanide (XXIa) (300).[122] The various salicylanilides, XXIa,[123] rafoxanide (XXIb)[124] and niclosamide (XXIc)[125] were evaluated in various clinical and field trials against intestinal worms, lung worms and liver flukes.



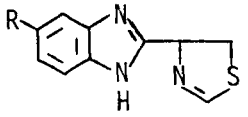
XXI

a: R_1=3,5,6-Cl_3, R_2=2-OH-3,5-Cl_2

b: R_1=3,5-I_2, R_2=3-Cl-4-(-O-  -Cl)

c: R_1=5-Cl, R_2=2-Cl-4-NO_2

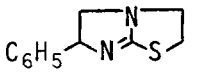
Capillariasis in man, caused by the round worm Capillaria philippinensis[30] was controlled better by thiabendazole (XXIIa) than by bithionol and bephenium.[126] Thiabendazole has been compared with levamisole, the levo-rotatory form of tetramisole (XXIII), and parbendazole (XXIVa) against natural helminthic infections in sheep.[127] Thiabendazole has been found useful in treating infestations of lagochilascaris.[128] Levamisole was found to be effective against lung worms in pigs,[129] it controlled nematodes in cattle with 94-99% efficacy against adult worms and 68-81% against larvae,[130] and in angora goats, 8 mg/kg was a safe dose, 16 mg/kg caused frequent drug intoxication and 64 mg/kg was generally fatal.[131] It is useful in worm infections in horses.[132] Parbendazole which is primarily effective against nematodes, was also found to be active against cestodes[133] and Trichinella spiralis in mice.[134] Mebendazole (XXIVb) is active against nematodes; it eradicates Enterobius vermicularis to the extent of



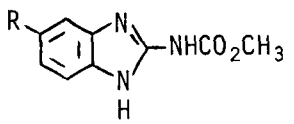
XXII

a: R=C_4H_9

b: R=$NHCO_2CH(CH_3)_2$



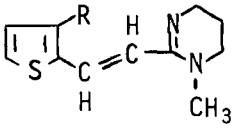
XXIII



XXIV

a: R=C_4H_9

b: R=C_6H_5CO



XXV

a: R=CH_3

b: R=H

90% with a single dose of 100 mg/kg and is active against Syphacia muris in rats.[135] Carbendazole (XXIIb) is approximately 3 times as effective as thiabendazole against nematodes. It is also effective at 5-15 mg/kg doses, p.o., in sheep against Haemonchus, Ostertagia, Trichostrongulus, Cooperia, Nematodirus, Strongyloides, Chabertia and Oesophagostomum.[136-138] Morantel (XXVa), at 6.25 mg/kg, eliminated 96-100% of Nippostrongylus and Nematospirioides compared with 76-89% efficacy for pyrantel (XXVb) in parallel trials.[139] The latter compound was 96.7% effective at 5 mg/lb against Ascarais lumbricoides; it was active against Necator americanus, inactive against Trichinella[140] but active in single 10 mg/kg doses in enterobiasis[141] and ascariasis.[142] 2-[2-(4-Hydroxyphenyl)-6-benzimidazolyl]-6-(1-methyl-4-piperazyl)benzimidazole (Hoechst 33258) is effective against microfilariae in cotton rats infected with Litomosoides carinii.[143] Quaternary ammonium salts of certain palmitoylaminoalkylamides are highly effective against the nematode Penagrellus redivivus.[144] Some 1-carbonyl-3-methyl-2-pyrazoline-4,5-dione-4-arylhydrazones are active against Trichinella spiralis.[145] Against microfilarial infections, 2-ethyl-6-methyl-2,3,4,4a,5,6,7,8-octahydro-1H-pyrazino(1,2-c)pyrimidine-1-one seems to be very promising.[146,147] O,O-Dimethyl-2,2,2-trichloro-1-hydroxyethylphosphonate, a choline esterase inhibitor, immobilizes the adult Onchocerca volvulus and consequently is useful in treating onchocerciasis.[148]

The antibiotic paromomycin (sulfate) cured 12 of 13 patients infected with the cestode Hymenolepis nana.[149] Cephamycin C (A-11884), an antibiotic isolated from Streptomyces limanii is active against Aspicularis tetraptera, Syphacia obvelata and Ascaris lumbricoides.[150]

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Section IV - Metabolic Diseases and Endocrine Function

Editor: I. J. Pachter, Bristol Laboratories, Syracuse, New York

Chapter 15. Prostaglandins and Related Compounds

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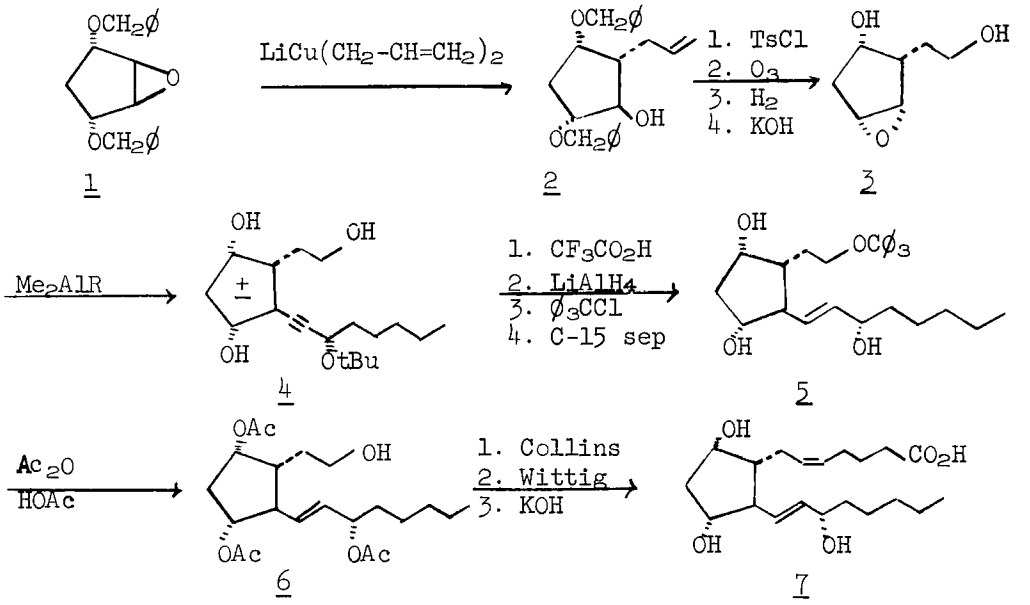
In reviewing a field which is growing as rapidly and in as many directions as prostaglandins, one must either limit the scope of the review or make it so long as to be unwieldy. In keeping with the former choice, this review will emphasize chemical developments in the prostaglandin area in 1971 (since the last review in this series[1]). A recent general review of prostaglandins is available[2], as well as comprehensive reviews of their chemistry[3], biochemistry[4], and pharmacology[5,6]. Vergroesen, et al[7] have summarized recent progress in clinical applications of prostaglandins while other reviews have focused on the relationship of prostaglandins with human reproduction[8-9] and fertility control[10].

I. Syntheses of natural prostaglandins - J. Fried and his associates[11] recently described a new synthesis of prostaglandins which utilizes metal-alkyl epoxide openings for the introduction of both side chains. Using diallyl copper lithium, epoxide 1[12] was converted in high yield to 2,

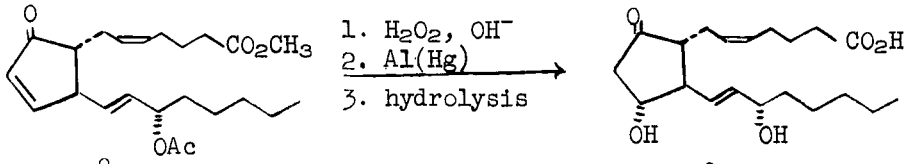


1 2 3 4 5 6 7

which via the 4-step process indicated above afforded diol epoxide 3. An important feature of Fried's synthesis is the regiospecific opening of epoxide 3 with dimethyl (S)-(-)-3-tert-butyloxy-1-octynylalane[12] leading

to a pair of diastereomers 4 in 65% yield (no attack at C-11). This regiospecificity is made possible by complexation of the alane with the ethanol side chain of 3 followed by internal delivery of the reagent to C-12. Chromatographic separation of 5 from the corresponding 15-epi-enantiomeric compound completed the resolution. Exclusive of the preparation and resolution of the optically active alane reagent, Fried's route affords $PGF_2\alpha$ (7) in 3% overall yield from 1 and is stereo- and regiospecific throughout.

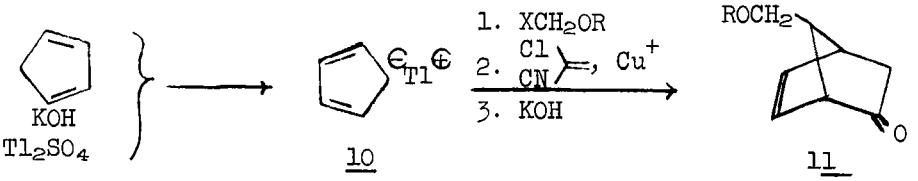
The discovery by Upjohn researchers[13,14] that natural [i.e. 15(S)] PGA_2, PGE_2 and esterified derivatives are found in the gorgonian coral, Plexaura homomalla (from various locations in the Caribbean area) has led to a short, efficient partial synthesis of PGE_2 and $PGF_2\alpha$[13,15]. 15(S)-PGA_2, acetate, methyl ester (~1.5% of the frozen wet weight of coral) was con-



8 9

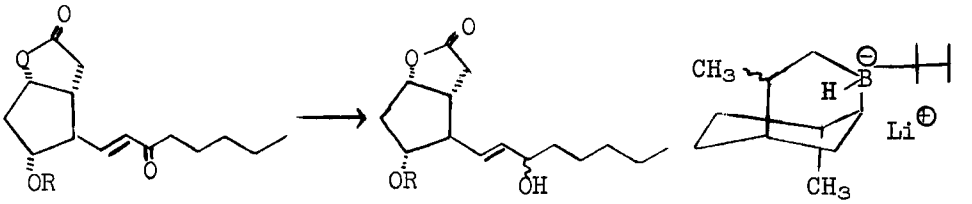
verted to an epimeric mixture of α,β-epoxyketones, reduced with aluminum amalgam, and following chromatographic separation of C-11 epimers, the PGE_2, 15-acetate, methyl ester was hydrolyzed enzymatically, thereby affording crystalline PGE_2 (9) in 50% overall yield from 8. Reduction of 9 gave pure $PGF_2\alpha$ in varying yield depending on the reducing agent. This represents improvement in both number of steps and overall yield over a similar process reported earlier[16] which utilized coral-derived prostaglandins with 15(R) configuration.

E. J. Corey has recently outlined a number of modifications of his 16-step synthetic route reported earlier[17]. Corey and his associates found that thallous cyclopentadienide 10 offered several advantages over



10 11

the commonly used alkali metal salts in the initial alkylation of cyclopentadiene[18]. Using this modification (and with X=Br, $R=CH_3$ above), 11 ($R=CH_3$) was isolated in 55% yield from cyclopentadiene. The use of a benzyl ether (11, $R=CH_2\phi$)[19] instead of a methyl ether offered an advantage for scale-up since its eventual removal by catalytic hydrogenation was experimentally simpler than the boron tribromide step required with the methyl ether[17]. Corey also found that reduction of 12 (R=p-phenylbenzoyl) with hydride reagent 15 gave a better ratio of 15α/15β reduction products than a large number of alternatives (different R, different hydrides)[19]. In addition to promoting a good 15α/15β ratio the p-



12 13, 15α-OH 14, 15β-OH 15

phenylbenzoyl group gave crystalline, easily separable reduction products (13,14).

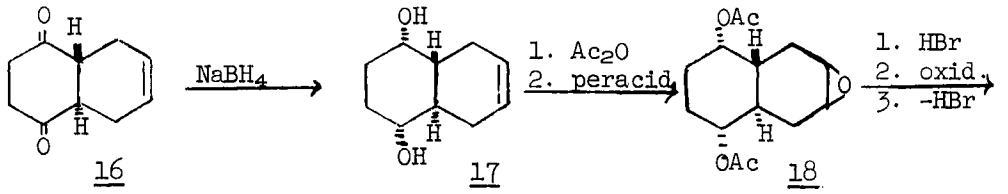
Hydride reagent 15 and lithium perhydroborophenalyl hydride[20,21] were both found to be effective for the stereospecific reduction of the PGE prostaglandins to the PGFα series[21]. Use of these reagents obviates the necessity of an PGFα/PGFβ chromatographic separation. Improvement in the selectivity of hydrogenation of the PG_2 to the PG_1 series[21] resulted from the use of hindered silyl ethers (at C-11 and C-15 in PGE_2) which render the C-13,14 double bond even less accessible than trimethylsilyl or tetrahydropyranyl ethers[22].

Ferdinandi and Just[23] reported the synthesis of dl-PGE_2 methyl ester and the corresponding 5,6-dehydro compound via a bismesylate solvolysis[24] route. The key step, the rearrangement of a cyclopropyl carbinyl system, was investigated in detail.

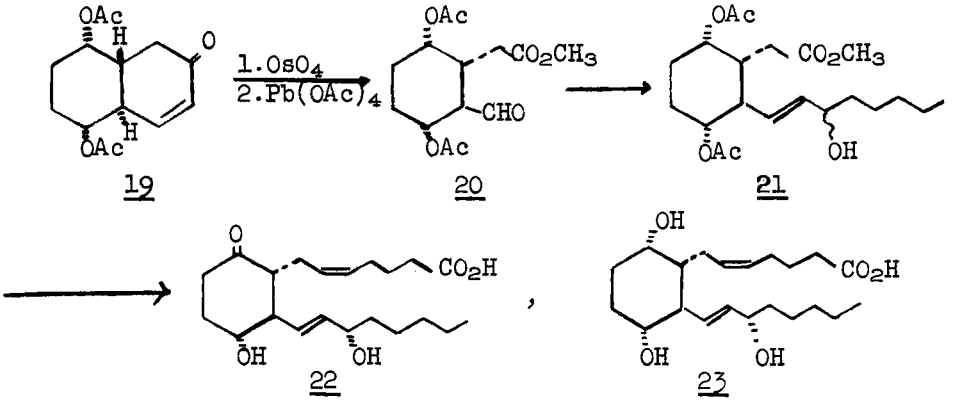
Miyano and his coworkers[25,26] have elaborated on their earlier synthetic route[27], starting with 3-oxo-undecan-1,11-dioic acid and styryl-glyoxal and have made dl-PGE_1, dl-$PGF_1\alpha$ and dl-13,14-dihydro-PGE_1 by this approach. While this route is fairly direct, it does require a number of complex separations of isomers.

Taub[25,28] has reported several improvements in the Merck prostaglandin synthesis[29] which allowed higher yields, a savings of several steps and incorporated a resolution procedure.

II. Syntheses of structurally modified prostaglandins - In the course of synthesizing prostaglandin analogs with a six-membered ring in place of the usual five-membered ring, N.S. Crossley[30] developed a new synthetic approach which is outlined below. Reduction of readily available[31] diketone 16 gave a mixture of products from which 17 crystallized in 24% yield. Conversion of the latter to crystalline aldehyde 20 was performed as indicated. (Epoxide 18 is the major product of an epimeric pair.)

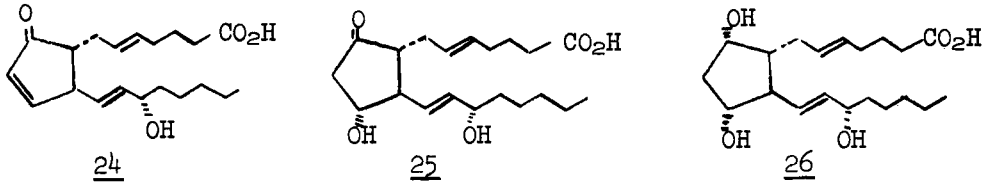


16 17 18



The remaining steps paralleled very closely those of the Corey synthesis[17] and finally afforded 22 and 23 which were "...less potent than the natural prostaglandins...in several biological assays..."[30]

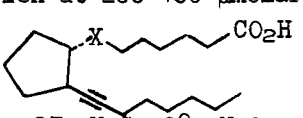
A new naturally occurring prostaglandin isomer, 5-trans-PGA_2 24, was isolated by Upjohn chemists[13,32] from the gorgonian Plexaura homomalla. The trans isomer content usually ranged between 5 and 15% of the PGA_2



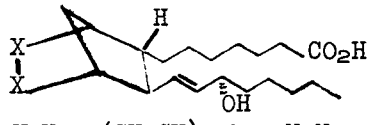
present. Using chemical transformations described earlier[13,15] 5-trans-PGA_2 was converted into 5-trans-PGE_2 25[33] (m.p. 76-77°) and 5-trans-$PGF_2\alpha$ 26 (m.p. 95-96°).

J. Fried[34] has reported the synthesis of (+)-7-oxa-$PGF_1\alpha$ and (+)-7-oxa-15-epi-$PGF_1\alpha$ and their enantiomers by a general route described earlier[12]. Of these four isomers, only 7-oxa-$PGF_1\alpha$ itself, in which all chiral centers possess the absolute configuration of natural prostaglandins, exhibits typical prostaglandin activity, while the others are either inactive or act as antagonists. Also only 7-oxa-$PGF_1\alpha$ was a substrate for prostaglandin 15-dehydrogenase[35] while the other three isomers were competitive inhibitors of this enzyme.

Another recently disclosed 15-dehydrogenase inhibitor is the thia-alkyne 27[11]. This competitive inhibitor exhibits a K_I=5.5 μmolar, while the corresponding oxa-analog 28 is a non-competitive inhibitor with 50% inhibition at 200-400 μmolar.



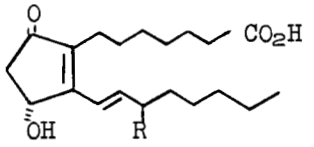
27, X=S; 28, X=O



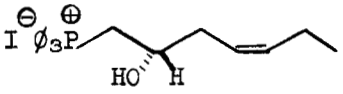
29, X-X = {CH=CH}; 30, X-X = {O-O}

The bicyclo[2.2.1]heptene derivative 29, prepared via a Diels-Alder reaction by Samuelsson and Corey[36], is a selective inhibitor of the biosynthesis of PGE_1 (but not $PGF_1\alpha$) from 8,11,14-eicosatrienoic acid. This derivative bears obvious resemblance to the endoperoxide 30, a proposed intermediate in prostaglandin E_1 and $F_1\alpha$ biosynthesis[37].

Microbial transformations of dione 31, yielding 15-epi-$\Delta^{8(12)}$-PGE_1 (32), were described by Miyano and coworkers[38]. Flavobacterium sp. NRRL-B-3874 reduced the (-) form of (±)-31 yielding (-)-32 (30%), while Pseudomonas sp. NRRL-B-3875 reduced the (+) form of the racemate stereoselectively producing (+)-32. The enantiomorph not used up in each reduction was optically active and could be separated chromatographically. In neither case was the corresponding 15-epimer formed.

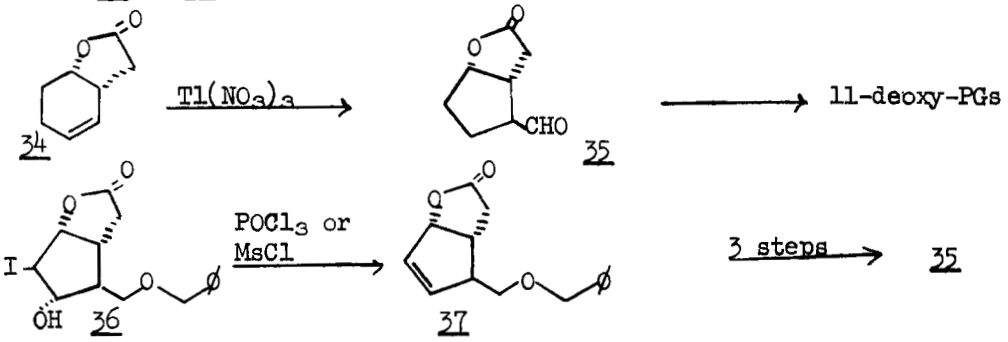


31, R=O; 32, R=β-OH



33

A number of analogs have been reported which were made by modifications of the Corey route[17]. Using the optically active Wittig reagent 33 (available in 7 steps from (S)-malic acid), Corey's group[39] prepared PGE_3 and $PGF_3\alpha$, identical with the same compounds of natural origin. 11-Deoxy prostaglandins have been synthesized by two groups. E. J. Corey[40] generated aldehyde 35 utilizing a modification of a thallium nitrate ring contraction reaction[41] (34→35), and converted 35 by the usual[17] procedure to 11-deoxy-PGE_2 and 11-deoxy-$PGF_2\alpha$. Crabbé and Guzmán[42] dehydrated Corey's iodohydrin 36 to 37, then by hydrogenation and oxidation obtained the same

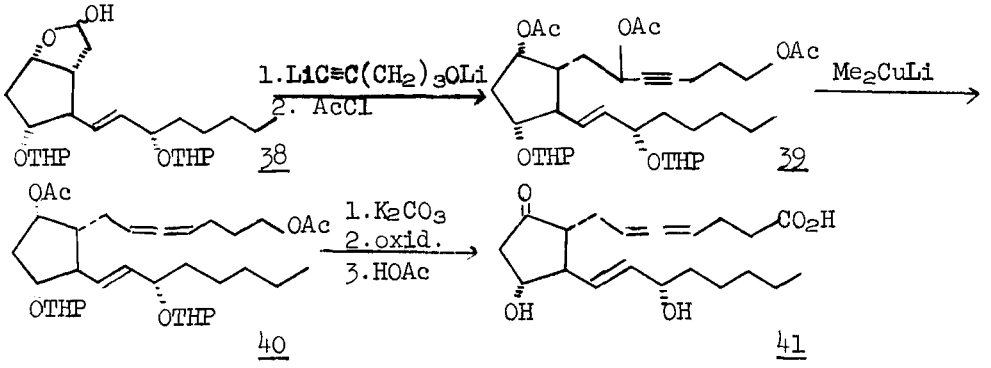


34 35 36 37 35

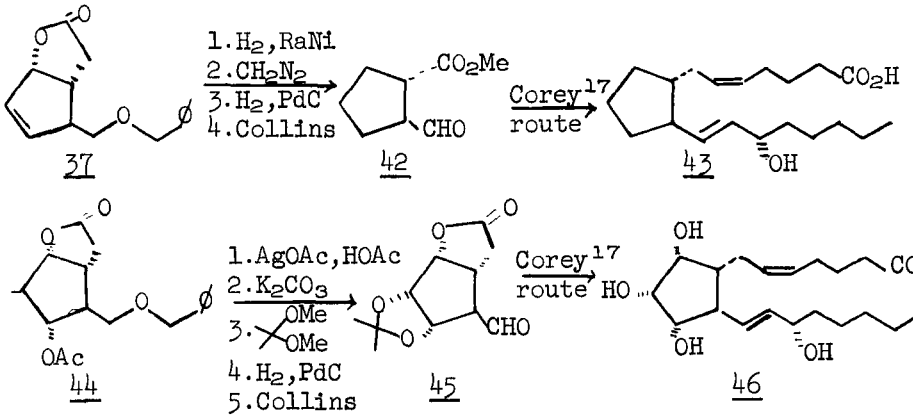
aldehyde 35. Corey and Grieco[43] accomplished the conversion of 36→37 using simply methanesulfonyl chloride and pyridine and then transformed 37 further without reducing the double bond, thereby affording a direct synthetic route to PGA's (i.e. avoiding the necessity of dehydrating PGE's). The biological activity of 11-deoxy-PGE_1 was reported several years ago[44].

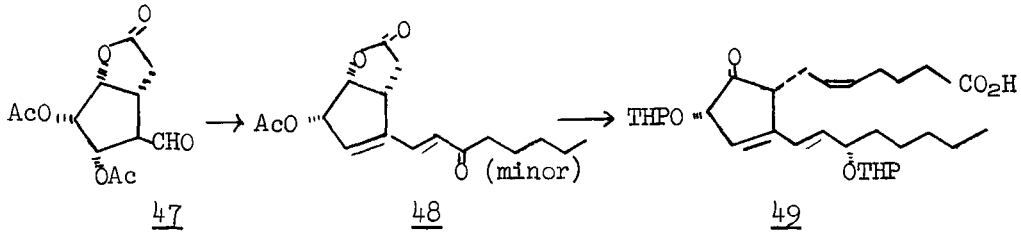
Crabbé and his coworkers[45] have synthesized analogs with an allenyl moiety in place of the 5,6-double bond (e.g. 41) as indicated below. The

key step (39→40) involved a reductive process followed by elimination



and rearrangement. Several additional classes of analogs were made by Crabbé[45] involving modifications of the five-membered ring. These include 9,11-bisdeoxy-PG's (e.g. 43), 10α-hydroxy-PG's (e.g. 46) and the acid labile derivative 49, prepared as indicated below:

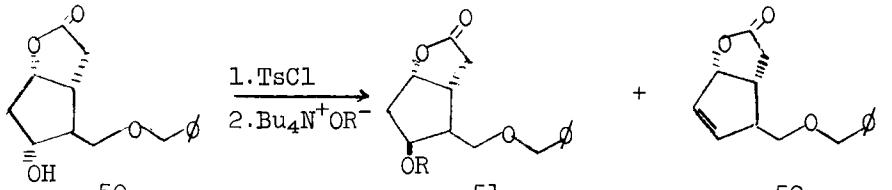




No biological data were disclosed for these analogs.

Upjohn chemists[13,46] recently disclosed an improved synthesis of 15-methyl-PGE_2 methyl ester. 15-Methyl-$PGF_2\alpha$ methyl ester[16] was converted to the C-11 mono-trimethylsilyl derivative, which on Collins oxidation and subsequent removal of the silyl group afforded 15-methyl-PGE_2 methyl ester in about 45% yield. Karim[47] has found that 15-methyl-PGE_2 methyl ester is at least 100 times more active than PGE_2 as a uterine stimulant in humans and its effects last at least three times as long as PGE_2.

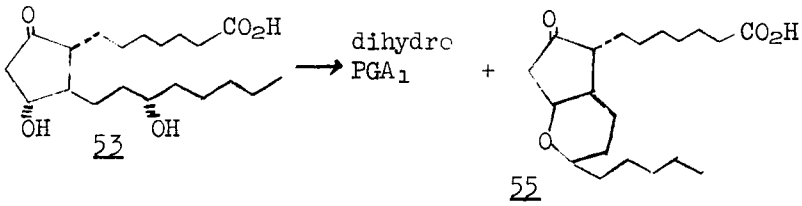
Corey[48] has reported a fairly efficient method for effecting inversion of configuration at C-11, thus making possible the synthesis of 11-epi-prostaglandins.



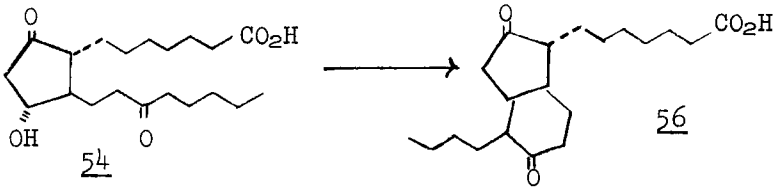
50 51 52

Treatment of the tosylate of 50 with tetrabutylammonium formate gave a 3:1 mixture of 51 (R=CHO) and 52. With acetate instead of formate, the ratio was 1.2:1 and with oxalate, only 52 was formed.

Merck chemists[49] reported the structures of several by-products formed in transformations of PGE_1. Hydrogenation of PGE_1 with 10% Pd/C in ethyl acetate gave, in addition to the expected 13,14-dihydro-PGE_1 (53), about 15% of the diketone 54. Formation of 54 implies that double



53 55



54 56

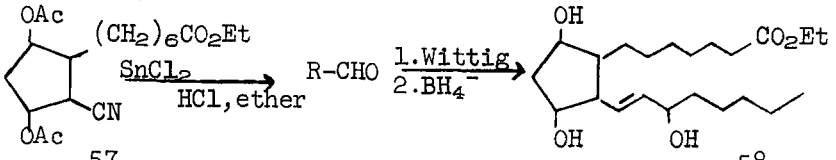
bond migration ($\Delta^{13} \rightarrow \Delta^{14}$) is competitive with hydrogenation under these conditions. Acidic dehydration of 53 gave dihydro-PGA_1 and ~35% of the cyclic ether 55 (from internal addition of the C-15 OH at C-11 of dihydro-PGA_1), while base treatment of 54 gave the Michael product 56.

A paper has appeared recently from the Unilever Laboratories[50] describing prostaglandin analog synthesis by bioconversion of a wide variety of synthetic polyunsaturated fatty acids. Prostaglandins formed in this manner included 2-trans-PGE, 3-trans-PGE, 4-cis-PGE, 5-trans-PGE_2, 18-cis-PGE. This work confirms the idea that not only the position of the double bonds, but also the chain length is an important factor for essential fatty acid activity. Also a close correlation is evident between EFA-activity and rate of prostaglandin formation in vitro.

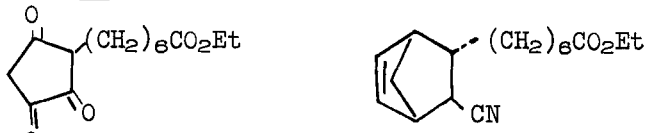
Pace-Asciak and Wolfe have elaborated[51,52] on the structure proofs

of several novel prostaglandin derivatives formed from arachidonic acid by rat stomach homogenates[51] and sheep seminal vesicles[52]. Preliminary accounts of this work appeared earlier[53].

Two approaches to the synthesis of the $PGF_1\alpha$ carbon skeleton, which merge in their final stages, have been reported by Katsube and co-workers[54,55]. The key intermediate in these routes is the nitrile 57 synthesized in one case[54] from trione 59 and alternatively[55] from the Diels-Alder product 60. These routes have the advantage of being short, but the



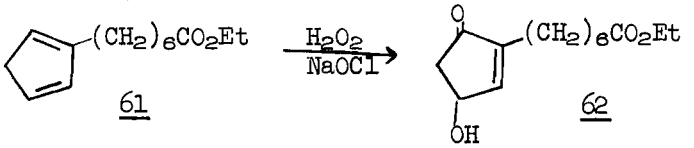
57 58



59 60

overriding disadvantage of being nonstereoselective at C-9, 11 and 15.

Sih, et al[56] recently disclosed the preparation of dl-15-deoxy-PGE_1 by a six-step sequence, the key features of which include the 1,4-



61 62

cycloaddition of chemically generated singlet oxygen (61→62) and addition of 1-lithio-trans-oct-1-ene (with Bu_3P-CuI, ether) to 62 to establish the 15-deoxy alkyl chain.

III. Assay, analysis - Chemical ionization mass spectrometry is a powerful analytical tool which has only recently[57] been applied to prostaglandin structure problems. The basis of this technique is the interaction between prostaglandin molecules and ions (CH_5^+, $C_2H_5^+$, $C_3H_5^+$) produced by electron impact on methane--a process that involves much lower energy than direct electron impact on the prostaglandins and hence results in less complex fragmentation patterns.

In the area of gas chromatographic analysis of prostaglandins, Axen, et al[58] prepared 3,3,4,4-tetradeutero-PGE_2 and -$PGF_2\alpha$ and used these as carriers in the glc-mass spec determination of PGE_2 and $PGF_2\alpha$ in picomole amounts. (Measure ratio of $\frac{M}{M+4}$.). This represents approximately a 10-fold increase in sensitivity over the glc-mass spec method reported earlier[59]. Pace-Asciak and Wolfe[60] illustrated the usefulness of cyclic n-butylboronates as glc derivatives of the PGF series. Using this derivative a mixture containing PGE_1, PGE_2, $PGF_1\alpha$ and $PGF_2\alpha$ may be analyzed

without prior class separation. Analysis of sub-nanogram levels of $PGF_1\alpha$ and $PGF_2\alpha$ (heptafluorobutyrate esters) was accomplished by glc with tritium electron capture detection[61]. A review of the various derivatives used for glc analysis of prostaglandins has appeared[62].

For the measurement of physiological levels of prostaglandins, radioimmunoassay remains the most sensitive technique. In addition to several such assays reviewed last year[1], K.T. Kirton[63] and H.R. Behrman[64] reported new immunoassays sensitive to picogram quantities of prostaglandins. Levine, et al[65] examined the specificities of antibodies against PGB_1, $PGF_1\alpha$ and $PGF_2\alpha$ and found that the cyclopentane ring appears to be immunodominant.

IV. Biology[4-6]- One feature of the biological area that deserves special mention here is the recent establishment of the connection between prostaglandins and anti-inflammatory drugs such as aspirin and indomethacin. Two British research groups have independently presented evidence that some of the therapeutic effects of aspirin and related drugs are due to the inhibition of prostaglandin synthesis. Vane[66] found that in cell-free homogenates of guinea pig lung, which will normally synthesize PGE_2 and $PGF_2\alpha$ on incubation with arachidonic acid, prostaglandin synthesis was inhibited by sodium aspirin, indomethacin, and sodium salicylate to an extent varying with the dose of the anti-inflammatory drug added. The concentrations necessary to produce this inhibition were clearly lower than the average therapeutic concentration in human blood. Smith and Willis[67] reported that these same drugs when added to resuspended human platelets inhibited thrombin-induced production of prostaglandins without impairing the "release reaction." A similar level of inhibition (80-95%) of prostaglandin synthesis was found in the blood from normal volunteers taken one hour after an oral dose of 600 mg of aspirin or 100 mg of indomethacin. Vane, et al[68] added further evidence in showing that indomethacin and aspirin block PGE_2 and $PGF_2\alpha$ production by isolated dog spleen.

Prostaglandins have been shown to induce fever and to be involved in inflammation--two conditions for which aspirin treatment is effective. (Vane has found no evidence for a link between inhibition of prostaglandin synthesis and aspirin's analgesic effects, however.) This work represents a start at unravelling the mechanism of action of aspirin and hence has extremely far-reaching implications. Vane[66] suggests that aspirin and indomethacin might be useful in the treatment of other conditions thought to be the result of prostaglandin release. Such conditions might include thrombosis, threatened abortion, rheumatism and IUD failures[69]. Tothill[70] found that several of the sedative drugs (e.g. desipramine, chlorpromazine, etc.) commonly used to prevent threatened miscarriage also prevent PGE_2 formation in isolated rat uterine strips, and he suggests that more effective therapy might be possible by giving aspirin in conjunction with the psychotropic agent. Collier[71] disclosed that the PGE and PGF content of human semen is reduced in subjects who are taking aspirin (possible cause of temporarily reduced fertility).

V. Clinical - No entirely new clinical applications of prostaglandins

have appeared in the last year. PGA_1 continues to be of interest as an inhibitor of gastric secretion[72] and for treatment of essential hypertension[73,74]. The hypotensive effects of PGE_1 have also been investigated in clinical situations[75]. At doses where the blood pressure effects of PGE_1 are minimal in healthy subjects, patients with essential hypertension exhibited decreases in blood pressure which started soon after infusion of PGE_1 and lasted 20-30 minutes. The cardiovascular effects of PGE_2 and $PGF_2\alpha$[76,77,78] as well as PGA_1, $PGF_1\alpha$ and $PGF_1\beta$[78] were studied in man with special emphasis on the incidence of side effects or cardiovascular effects as a function of dose and mode of administration.

The greatest volume of clinical prostaglandin research still appears to be directed toward the areas of reproduction and fertility--labor induction, therapeutic abortion and contraception. Several groups have substantiated and extended the reports reviewed last year[1] that intravenous administration of either PGE_2 or $PGF_2\alpha$ is effective for terminating pregnancy[79-83]. While effective, therapeutic abortions by this route are accompanied by a high incidence of vomiting and diarrhea. The side effects found in the intravenous route can be minimized using intra-uterine administration[84] (lower doses required). Intra-amniotic injection of PGE_2 or $PGF_2\alpha$ also overcomes these problems and has been used effectively[85-87]. Intravaginal administration is useful both for therapeutic abortion and labor induction[88-89]. Several groups[80,82,87] have suggested that the primary use of prostaglandins for therapeutic abortion may be in the second trimester where alternative methods are difficult or hazardous (e.g. hypotonic saline or glucose, hysterotomy).

For labor induction, all of the aforementioned modes of administration of prostaglandins have been used successfully[1,88,90,92]. In addition, orally administered PGE_2 and $PGF_2\alpha$ are effective for labor induction[93], whereas the larger doses required for orally induced therapeutic abortion produce marked levels of gastrointestinal side effects[93]. Karim showed that the action of prostaglandins on the human uterus is not mediated through the release of oxytocin[92].

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Chapter 16. Atherosclerosis

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INTRODUCTION

Atherosclerosis is an extremely common disease of the large arteries, which constitutes the pathologic basis for most coronary heart disease, stroke, aneurysm and occlusive arterial disease.[1] A report has been published[2] which conveys the magnitude of the health problem associated with atherosclerosis, and reviews the evidence for identification of risk factors (major risk factors: hypercholesterolemia, hypertension and cigarette smoking). A program is outlined for control of risk factors utilizing all available procedures. The statement on drugs indicates a need for continued evaluation of currently available drugs and for development of new ones, with emphasis on safety.

Basic pathology of atherosclerosis is well described in a text,[1] and an important monograph[3] provides a broad coverage of the disease, from etiology to clinical management. An older, though still valuable, monograph[4] deals with the evolution of the atherosclerotic plaque. A comprehensive picture of current research in atherosclerosis can be obtained from the recently published Proceedings of the Second International Symposium on Atherosclerosis.[5] Much of the material contained in this book has not yet appeared in journals.

BIOLOGICAL RESEARCH

Lipoprotein Metabolism - Previous reviews have indicated that major research effort has been directed toward development of diets, surgical procedures and drugs which lower elevated blood lipids. The reasons for this emphasis on blood lipid control are detailed elsewhere.[2,3,5] Blood coagulation and platelet aggregation, areas with important relationships to atherosclerosis, will be discussed in chapter 8.

The lipids of plasma exist as components of lipoproteins, and it is these which must be considered as potential pathogenic agents, since ultimately it is the physicochemical and biological properties of these complex molecules which determines whether or not a given lipid will reach the arterial subintima and be deposited there. On the basis of this reasoning, studies have been conducted to determine the atherogenic potential of the various lipoprotein classes, starting with the work of Gofman and coworkers.[6] For recent concepts the reader is referred to the classic series of papers by Fredrickson and coworkers,[7] and to recent reviews.[8,9] Familiarity with the Fredrickson classification of hyperlipoproteinemias will be assumed in the following discussion.

Large elevations of low-density lipoproteins (LDL), such as occur in homozygous type II hyperlipidemia are profoundly atherogenic;[10] the situation is less clear for slight or moderate elevations of LDL. A

proposed relationship of plasma triglyceride elevation to coronary heart disease[11] implies atherogenicity of the triglyceride-rich very low-density lipoproteins (VLDL). VLDL also contain cholesterol esters and free cholesterol, as well as LDL apoprotein (see below).

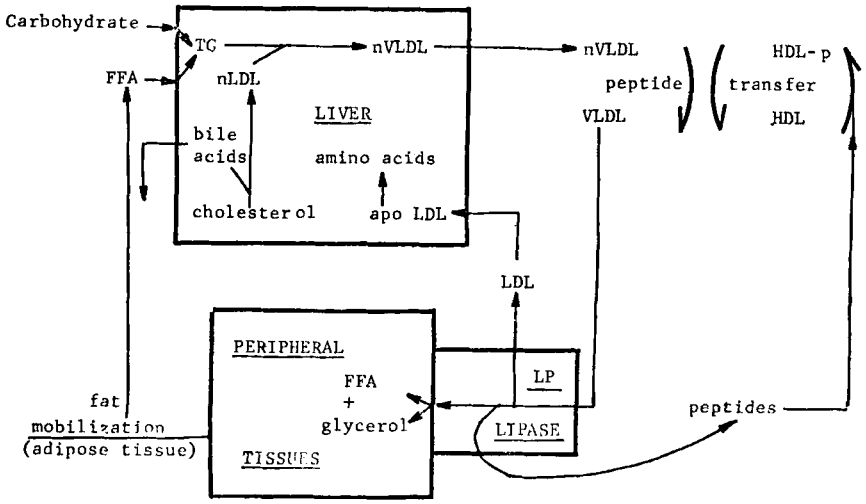
An analysis has been reported[12] of relationship of lipids and lipoproteins to development of coronary heart disease (CHD) over 14 years in 5127 men and women in the Framingham Study. Serum cholesterol, LDL and VLDL showed strong statistical discriminatory power for development of CHD, VLDL somewhat less than the other two. In men of all ages and in young women VLDL did not predict CHD when its cholesterol risk factor was statistically removed. In women of 54-69 years, however, VLDL appeared to be the important risk factor, rather than cholesterol. With the exception of these older women, the data seem to indicate that cholesterol, presented to the artery either as LDL or VLDL, is the pathogenic agent. No protective role could be imputed to serum total phospholipid, since the cholesterol/phospholipid ratio showed no ability to discriminate for or against CHD. The analysis has been criticized on the basis of the basic data.[13]

Two groups have attempted to correlate CHD, documented by coronary arteriography, with hyperlipoproteinemia and other risk factors. Bemis et al[14] measured lesion progression by repeated arteriography and found progression to be strongly correlated with hyperlipidemia (cholesterol and /or triglyceride). Although the numbers are small, types III and IV hyperlipoproteinemia were better correlated to lesion progression than type II. In a study of 50 consecutive hospital admissions for CHD, Salel et al[15] determined the prevalence of various risk factors. The highest prevalence by far was for male sex; type IV hyperlipoproteinemia was next, and type II a poor third. Particularly important was the finding that prevalence of type IV was significantly greater in the CHD group than in a control population, while prevalence of type II was not significantly different for the two populations. The lower prevalence of type II in the total population may require a larger sample than was used. These studies represent early efforts to determine atherogenicity of LDL (type II) and VLDL (type IV) elevations. Much more needs to be done. It appears at this point that both LDL and VLDL should be regarded as atherogenic, but that quantitative comparisons cannot be made.

A general picture of lipoprotein metabolism has now evolved, with several important details added during 1971. Triglyceride (TG) is synthesized in the liver when fatty acids become available, either from local synthesis or from the blood (mobilization from adipose tissue). Formation of VLDL is an immediate consequence of TG production in the hepatocyte.[16-18] Before entering the circulation the nascent VLDL differs in protein composition from VLDL isolated from the circulation,[17] containing relatively more of the peptide characteristic of LDL (apo-LDL) and less of peptides found in high-density lipoprotein (HDL). Evidence has been obtained that peptides are transferred reversibly from HDL to nascent VLDL in the circulation.[19,20] Two such transferrable peptides have been identified, designated according to C-terminal amino acid as

apoLP-glu[20] (R-glu[21]) and apoLP-ala (R-ala). Functional roles for these peptides are strongly suggested by the findings that apoLP-glu activates the enzyme lipoprotein lipase, and that apoLP-ala can activate, or, depending on concentration, inhibit it.[22-24] Lipoprotein lipase is the enzyme which removes TG from VLDL by hydrolysis at sites of tissue TG utilization. During hydrolysis of VLDL-TG, LDL, apoLP-glu and apoLP-ala are released.[20] LDL, labeled in the protein moiety with ^{125}I, was not reutilized for VLDL synthesis.[25]

An overall scheme for triglyceride transport can now be visualized[19] with roles assigned to all of the lipoprotein classes. While other functions of the plasma lipoproteins certainly exist, there is a good probability that their triglyceride transport function is a major determinant of their plasma concentrations.



nVLDL=nascent VLDL; HDL-p=HDL with transferrable peptides attached; FFA= free fatty acids. Symbols and arrows outside of boxes represent transport in blood plasma.

* *

According to this scheme, type IV hyperlipidemia (elevated VLDL) could be the result of hepatic overproduction of TG or of decreased peripheral utilization of VLDL-TG. Type II could be due to accelerated TG turnover, exceeding a normal capacity to degrade LDL formed by hydrolysis of VLDL-TG, or to decreased ability to degrade LDL. Recently a subtype II, type IIb, has been recognized,[26] in which both LDL and VLDL are elevated. This subtype commonly is accompanied by obesity,[27] and it may be that triglyceride overproduction has exceeded the capacities of both the TG removal system and the LDL degradation system in these patients. Levy[25] has shown that cholestyramine increases the fractional catabolic rate

of ^{125}I-labeled LDL, without affecting its endogenous production rate. Since this drug is the most effective for treatment of classical type II (type IIa), it may be inferred that LDL degradation is faulty in this disorder. Levy also found that nicotinic acid, which is also effective in type II, decreased synthesis of LDL without affecting the rate of its catabolism. Combination of cholestyramine with nicotinic acid produced synergism in reduction of cholesterol.[25,28,29]

Using a similar rationale, Howard and Hyams[30] found that a combination of clofibrate and DEAE-Sephadex synergized in reducing plasma cholesterol of type II patients. These patients had failed to respond to clofibrate alone, and responded modestly (13% cholesterol reduction) to DEAE-Sephadex alone, but experienced an average cholesterol reduction of 33% on the combination. By increasing the combination dose of DEAE-Sephadex from 12 to 18 gm/day, cholesterol reduction could be increased to 40%. Surprisingly, combination of cholestyramine with clofibrate was not as effective as the clofibrate-DEAE-Sephadex combination. White[31] has shown that clofibrate cannot prevent the increase in hepatic cholesterol synthesis occurring during cholestyramine administration to the rat.

Studies at the NHLI have cleared up some of the mystery surrounding type III hyperlipidemia (broad-β disease). In type III patients, VLDL is partially degraded at a normal rate to an intermediate lipoprotein[32] with β-mobility. This lipoprotein accumulates, and is only slowly degraded in these individuals, due to a deficiency of one component of lipoprotein lipase.[33] This result implies that VLDL degradation occurs in at least two stages, one of which is defective in type III hyperlipidemia. Other evidence suggestive of this comes from studies of lipoprotein lipase (LPL) activation by the HDL peptides.[34]

Consideration of the TG transport scheme offers an explanation for the observation that heparin treatment, while reducing circulating VLDL, often increases plasma LDL.[35,36] Heparin releases LPL into the circulation, where it hydrolyzes TG from VLDL and chylomicrons. The released fatty acids must be partially reutilized by the liver for synthesis of TG, and consequently VLDL. Turnover of VLDL is thus increased, with an obligatory increase in LDL synthesis. It seems better to attempt to increase the TG-clearing mechanism at its physiological site, the tissue capillary endothelium, where the released FFA are transferred into tissue.[37] Glueck[38] has reported that oxandrolone, an anabolic-androgenic steroid, reduced plasma VLDL and chylomicrons in types III, IV and V, and produced striking increases in LPL enzymes which could be released into the circulation by heparin. Such increases probably reflect increased amounts of enzyme at the tissue capillary site. LDL values were not reported in this study, but cholesterol levels, most of which were originally in the normal range, showed little change. Similar effects are reported[39] for the progestational steroid norethindrone acetate.

Obesity and Cholesterol Metabolism - A relationship between obesity and atherosclerosis has been suspected and proposed[40] on an empirical basis, but little understanding has evolved, relative to the potential importance

of such a relationship. In particular, no consistent relationship has been found between plasma cholesterol and obesity.

Schreibman et al[41] studied cholesterol kinetics in very obese patients with normal cholesterol levels, and found cholesterol turnover rates 2 1/2 times normal. Excretions of both neutral sterols and bile acids were increased. Analysis of plasma labeled-cholesterol decay curves showed normal size of the readily exchangeable cholesterol pool (pool A), but the slowly exchanging pool (pool B) was doubled. The extra mass of cholesterol in pool B could be accounted for by the cholesterol content of excess adipose tissue.

In the rat, Angel and Farkas[42] reported that cholesterol synthesis from glucose occurs at about one-fifth the rate in adipose tissue as in liver, per unit weight of tissue. Because of the greater mass of adipose tissue, it ranked with liver as a cholesterogenic organ. Under conditions of dietary cholesterol intake, cholesterol synthesis in adipose tissue was suppressed much less than in liver, and adipose tissue became a principal endogenous source of cholesterol.

These studies show that adipose tissue serves as a potentially important site of cholesterol production and as a reservoir of slowly mobilizable cholesterol. In spite of this, the very obese patients of Schreibman et al maintained normal plasma levels of cholesterol. It is hard to see how obesity posed a threat to the arteries of these patients, at least via cholesterol, in a steady state of body weight. Conceivably, rapid loss of body weight could mobilize large quantities of cholesterol and raise the plasma concentration until a new steady state was established. Obese persons might exhibit resistance to hypocholesteremic drugs by virtue of an ability to compensate, out of adipose tissue stores, for decreased cholesterol synthesis or increased excretion. Ahrens[43] has observed maintenance of elevated plasma cholesterol in the face of net loss of body cholesterol during clofibrate therapy, but did not report that this was related to obesity.

Atherogenesis - In spite of the high probability that infiltration of lipoproteins into the arterial wall is an essential event in atherogenesis, there is relatively little understanding of the mechanisms of subsequent events: lipid deposition, intimal proliferation, smooth muscle cell migration to the subintima, foam cell formation, etc.

It is known that low-density and very low-density lipoproteins can form complexes with acid mucopolysaccharides (AMPS), prominent components of early lesions. Such complexes have now been isolated from early human lesions by Berenson et al,[44] and shown to include both LDL and VLDL complexes, with predominance of LDL. The AMPS involved were chondroitin sulfate and heparitin sulfate.

The elastic lamellae, which provide elasticity to the arterial wall, figure prominently in the early phases of development of the atherosclerotic lesion. They are sites for microscopically visible lipid

deposition and undergo fragmentation and disruption. Kramsch et al[45] have published a study of elastin composition in normal and atherosclerotic human aortas. Striking increases in lipid content of elastin were correlated with severity of atherosclerosis. Eighty per cent of the lipid in elastin from normal tissue and from plaques was cholesterol, present chiefly as esters. Concomitant with the increase in lipids in atherosclerotic samples, there were an increase in polar amino acids in elastin and a decrease in cross-linking amino acids. Using a different isolation procedure, Yu[46] also found an increased content of polar amino acids in elastin from atherosclerotic aortas, but essentially no change in cross-linking amino acids. Robert et al[47] have discussed the biochemistry of elastin as related to aging and atherosclerosis. The high elastin content of arteries may account in part for their extraordinary ability to take up cholesterol from the circulation.[48] Uptake of lipids by elastin may partially denature the protein, rendering it more susceptible to hydrolysis by elastase and other proteases derived from platelets or phagocytes.[47]

Lipid-filled cells, "foam cells," are a prominent feature of the atherosclerotic lesion, and contribute to the core of cellular debris, the so-called "gruel" of advanced lesions. This material can embolize directly from ruptured plaques or serve as a thrombogenic focus. The origin of these cells has been disputed, although considerable evidence has accumulated recently that they derive from smooth muscle cells. The contribution, if any, of circulating macrophages to this pool of cells has remained unsettled. Cookson,[49] in an electron microscopic study of lesions in cholesterol-fed rabbits, confirmed the contribution of smooth muscle cells, and obtained evidence that circulating macrophages play a prominent role in phagocytosis of lipid in the lesion, and in formation of debris by rapid degeneration. Wurster and Zilversmit,[50] in a study of phagocytic activity of peritoneal macrophages from cholesterol-fed rabbits, concluded that this type of cell probably does not contribute importantly to lipid accumulation in the aorta. The two studies are not necessarily incompatible, but further work will be required to settle this problem. Cookson suggests that the activity of the smooth muscle cell is essentially reparative, and should be stimulated to promote healing of the lesion. Dzoga et al[51] have reported that hyperlipemic serum, or LDL isolated therefrom, from cholesterol-fed rabbits or monkeys stimulates growth of homologous arterial smooth muscle cells in tissue culture. LDL from normal animals did not have this effect at the same concentration.

Cholesterol accumulated in atheromatous lesions is largely esterified with fatty acids, especially oleic acid. The change in fatty acid composition of cholesterol esters between circulating lipoproteins and atheromas indicates an esterification process operative in the developing lesion. Kothari et al[52] have demonstrated enzymes in normal artery which catalyze both synthesis and hydrolysis of cholesterol esters. Reaction rates of esterification and hydrolysis were found to be strongly dependent on the physical state of the reactants: an emulsified state promoted esterification, whereas a micellar state promoted hydrolysis.

Atherosclerotic tissue is reported to have a higher cholesterol esterification rate than normal arterial tissue,[53,54] as well as a greater permeability to cholesterol esters bound to lipoprotein.[54,55]

Caro et al[56] have published a new hypothesis relating shear at the blood-wall interface to removal of cholesterol from the artery wall. An assumption that cholesterol synthesis in the wall is an important source of lesion cholesterol is probably invalid, but could be replaced by an assumption regarding cholesterol esterification. The hypothesis is intended to account for the pattern of distribution of human lesions.

Estrogens and other Hormones - Evidence from clinical trials indicates that conjugated equine estrogens,[57,58] but not ethinyl estradiol[58,59] or Anvene,[58,60] protect against death from coronary heart disease in survivors of myocardial infarction. Dissociation of the protective effect from changes in blood cholesterol was observed,[58,59] and large doses of equine estrogens, sufficient to alter blood lipids, resulted in excess cardiovascular mortality,[57,61] especially during the early post-infarction period.[57]

Wolinsky[62] has shown that hypertension produces increases in aortic thickness, cellular protein, elastin and collagen, and that these changes reverse in female rats when the blood pressure is returned to normal. The changes did not reverse in males, but estradiol treatment of males during the hypertensive phase prevented the hypertrophic changes.[63] Progestin treatment slightly enhanced the effects of hypertension. Fischer has reported[64] that estradiol replacement in ovariectomized rats increased turnover of elastin and collagen in aorta, decreased total aortic collagen and decreased collagen-to-elastin ratio.

These studies strongly suggest that estrogens reduce arterial responses to injury, which are important in development of the atherosclerotic lesion, specifically cellular proliferation and laying down of connective tissue fibers. Such effects, if limited, could retard the chronic atherogenic process, but they could be harmful in circumstances requiring strengthening and repair of severely diseased tissue, especially weakened walls of arteries and heart. The potential importance of these findings requires their confirmation, preferably in another species in which overall catabolic effects of estrogens are less pronounced.

Marked hypocholesterolemic effects of chronic glucagon administration have been described for rats in a variety of dietary and endocrine situations.[65] The data indicate that this hormone must be taken into account in evaluating effects of endocrine manipulation on blood cholesterol.

DRUGS

Trials of Drug Efficacy - Dayton[66] has delineated the need for hypolipidemic drugs as part of a total program for lipid control in atherosclerosis. Although the rationale for use of such drugs is strong, final proof of

efficacy must come from unequivocal evidence of prevention or reversal of human atherosclerosis and/or its complications. With the advent of clofibrate as an active hypolipidemic drug, several studies were initiated. Results from two of these have now been reported. Both studies (designated the Newcastle[67] and Scottish[68] studies) fall in the category of secondary prevention trials, i.e. they were designed to determine the effects of treatment on progress of atherosclerotic disease manifestations in patients established as having the disease at the onset. Although differences between the studies exist, leading to some differences in findings, certain important and unexpected results were common to both studies. Clofibrate was found to protect against death and new myocardial infarction in those patients exhibiting angina as a manifestation of their disease, but significant protection was not afforded to patients whose manifestation was previous myocardial infarction without angina. Among the clofibrate-treated subgroups in the Newcastle study, there was no relationship between cholesterol reduction and protection against cardiovascular death or myocardial infarction, although such a relationship obtained in part of the Scottish study.

These results suggest that the major protective effect of clofibrate in these groups of patients may not have been mediated by reduction of blood lipids. Although the subjects were chosen for their manifest (and therefore advanced) disease and might be expected to respond relatively poorly to lipid reduction, this conclusion is disturbing. The authors of the Newcastle study suggest that clofibrate somehow protects against dysrhythmia, a common cause of death in angina patients.

Clofibrate - Space does not permit discussion of the many publications on this drug. Some of the more important ones dealt with effects on cholesterol synthesis,[69,70] cholesterol and bile acid excretion,[71] triglyceride synthesis,[72,73] and effects on cell ultrastructure.[74] For general information, two sources are recommended.[75,76]

Investigational Drugs - A number of clinical publications on nafenopin (SU-13,437, Melipan) appeared during the past year.[77-83] In general, results confirm previous reports of greater reductions of serum triglycerides than of serum cholesterol. Early claims for an advantage over clofibrate in treatment of type II hyperlipidemia have not been confirmed. In a comparative study of the two drugs, Dujovne et al[77] found nafenopin (600 mg per day) slightly, but not significantly superior to clofibrate (2 gm per day) in overall reduction of serum lipids, but cholesterol reduction was not superior in type II, and there may have been an "escape" of serum TG in the type IV patients with nafenopin. Incidence of liver function abnormality was higher on nafenopin than on clofibrate, but clofibrate produced a significant elevation of serum creatine kinase (usually related to muscle damage), whereas nafenopin did not. Hartmann,[78] Manucci et al[79] and Fellin et al[80] found Type II hyperlipidemia less responsive than the hypertriglyceridemias to nafenopin; all reported no, or minimal, effects on liver function, but deGennes and coworkers[81] reported four cases of jaundice "of unknown mechanism" in a series of 126 patients. Manucci et al found nafenopin to lower elevated plasma fibrinogen levels in hy-

perlipidemic patients, but to increase plasma plasminogen and decrease euglobulin lysis time (the latter two indicating a possible reduction in ability to lyse clots). No effect was seen on platelet adhesiveness to glass. Nafenopin is reported to increase hepatic fatty acid synthesis in the mouse with little effect on cholesterol synthesis.[84] A finding of liver pathology in long-term, high-dose studies in rats has resulted in withdrawal of the IND for nafenopin.[82]

Halofenate (MK-185, 2-acetamidoethyl (p-chlorophenyl) (m-trifluoromethylphenoxy) acetate) is a hypolipidemic compound with a consistent hypouricemic effect.[85-88] In rats, halofenate reduced both cholesterol and TG, with a potency 5.7 times that of clofibrate,[89] but in man it has demonstrated little ability to reduce serum cholesterol, although it is about twice as potent as clofibrate, but no more effective, in reducing serum TG.[86-88] Halofenate is reported to displace thyroxine from plasma thyroxine binding globulin,[85] thereby reducing protein-bound iodine; there is no apparent effect on clinical thyroid status. Serum uric acid levels are lowered through a uricosuric mechanism.[85] Although the compound, like clofibrate, causes liver enlargement in rats,[89] no abnormalities of liver function were reported. Serum creatine kinase was elevated, and the greatest elevations were associated with gastrointestinal symptoms.[85]

Removal of bile acids via increased fecal excretion has been accomplished through use of bile-acid binding resins, and by ileal by-pass operations. Both procedures have been effective in reducing serum cholesterol levels in patients with the heterozygous form of type II hyperlipidemia, but much less so in the homozygous form.[90,91] Grundy et al[92] have examined the dynamics of cholesterol and bile acids in type II patients receiving cholestyramine or undergoing ileal exclusion. They found the two treatments equivalent, even quantitatively. Generally, the operative procedure has been more effective.[90] Grundy et al found that bile acid removal led to increased cholesterol synthesis, probably both in liver and intestine, and that this compensated for the bile acid loss in some cases, in which plasma cholesterol did not decrease, or even increased. Kuo[93] has reported that the frequently observed elevation of plasma VLDL during cholestyramine therapy can be controlled by carbohydrate and calorie restriction. Colestipol (U-26,597A), a copolymer of tetraethylenepentamine and epichlorhydrin, has been reported to be similar to cholestyramine in potency[94,95] and tolerance.[95] Combinations of bile acid-binding agents with other drugs are discussed under lipoprotein metabolism in this review.

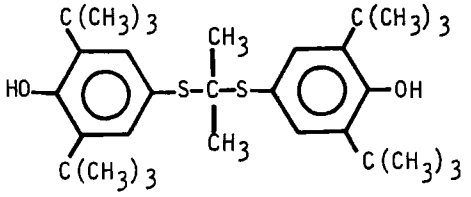
The mechanism of the hypocholesteremic effect of neomycin has been reviewed and investigated by Thompson and coworkers.[96] They found that this basic antibiotic caused precipitation of cholesterol, fatty acids and bile acids from the micellar phase of aspirate from the small intestine, following administration with a test meal. Disruption of mixed micelles of these substances could be expected to interfere with absorption, especially of cholesterol. Lipase activity was not affected. Samuel[97] has reported a strong correlation between the ability of neomycin to prevent fecal conversion of cholic acid to deoxycholic acid and its ability to

lower plasma cholesterol in hypercholesteremic children.

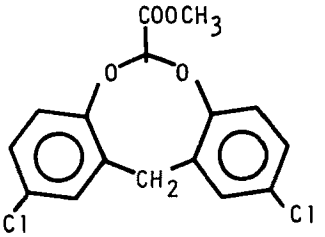
Miscellaneous and New Drugs - Kritchevsky[98] has reviewed recently developed hypolipidemic drugs. Cardiac tolerance to D-thyroxine (dextro-thyroxine, D-T_4) is reported to be improved by concomitant administration of the β-blocking agent, propanolol.[99] A clinical report[100] indicates that D-triiodothyronine (D-T_3, dextro-triiodothyronine) is an effective hypolipidemic agent with minimal cardiac side effects. A new thyroxine analog, D,L-α-methylthyroxine ethyl ester (CG-635) has been studied in Germany.[101-103] The compound is reported to have improved ratios of hypocholesteremic effect to effects on O_2-consumption, heart weight and heart rate in the rat, in comparison to the T_3 and T_4 isomers.[102] The clinical dose is relatively large (40 mg/day) and PBI increases markedly.[103] CG-635 lowered plasma cholesterol moderately in types II and IV patients, and triglycerides in type IV.[101-103] Some effects on liver function were noted.[101]

Probucol - (DH-581, Biphenabid) was shown to produce moderate reductions in plasma cholesterol in several clinical studies;[104-107] triglycerides were not reduced, and in some cases were increased.[104] Mechanism of the hypocholesteremic effect remains in doubt, but may involve inhibition of release of lipoprotein cholesterol from the liver.[108] Kritchevsky et al[109] found probucol to reduce plasma cholesterol and atheroma formation in cholesterol-fed rabbits. Probucol apparently was well tolerated in the human studies.

Treloxinate - (methyl 2,10-dichloro-12H-dibenzo[d,g][1,3]dioxocin-6-carboxylate) is a new hypolipidemic agent of the general clofibrate class.[110,111] The p-chlorophenyl substituents are held in a fixed conformation as part of the dioxocin ring structure. In rats treloxinate is 8 times as potent as clofibrate in reducing plasma cholesterol, and 30 times as potent in reducing triglycerides. Treloxinate inhibited hepatic synthesis of cholesterol from acetate, but not from mevalonate in the rat, and increased oxidation of cholesterol-26 ^{14}C to $^{14}CO_2$. In hypothyroid dogs, it markedly reduced LDL cholesterol, but not HDL cholesterol.



Probucol



Treloxinate

D-N-(α-methylbenzyl) linoleamide is the most potent of a series of N-substituted linoleamides which prevent hypercholesteramia and atherogenesis in cholesterol-fed rabbits.[112] The compound does not affect cholesterol synthesis or side-chain oxidation in vitro, but appears to inhibit cholesterol absorption.[113] This effect may be related to its ability to form a complex with cholesterol.[114] The D-isomer is more slowly metabolized than the L-isomer, which may account for its greater activity.[115] Some simple mono- and di-substituted nicotinic acids are reported[116] to be more potent than the parent compound in inhibiting cholesterol and fatty acid synthesis in rat liver.

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Chapter 17. Steroids and Biologically Related Compounds

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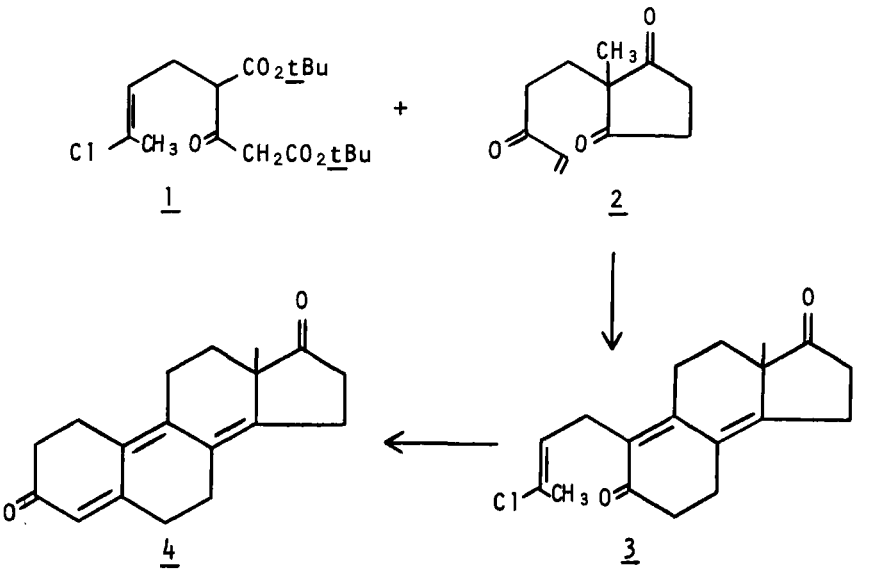
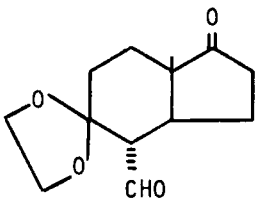
Introduction - The flow of publications in the area of steroid research continued unabated in 1971. However, in much of this the rigid steroid skeleton served solely as a convenient framework for investigation of the stereochemistry of new reactions. Many other papers dealt with the isolation and identification of new sterols from natural sources, while others were reports of new derivatives which seemed to us to have little prospect for practical utility. In this review we have selected primarily those articles which appeared to have particular interest for the medicinal chemist.

Total Synthesis - Several new procedures for the total synthesis of steroids have been developed. A novel synthesis of a 19-nor steroid was reported in which tert-butyl acetonedicarboxylate was alkylated to give 1, which was condensed with the disubstituted cyclopentanedione 2 to give the tricyclic intermediate 3. Hydrolysis and ring closure afforded 4 in 34% overall yield.[1] The utility and convenience of this procedure may permit it to be adapted to the syntheses of a large variety of useful steroids and steroid intermediates.



Two new total syntheses of dl-estrone utilize the bicyclic aldehyde 5[2] and the vinylketone 6[3] as key intermediates. These are condensed respectively with m-methoxybenzyltriphenylphosphonium chloride and 2-methylcyclopentanedione to give the appropriate intermediates for ring closure.



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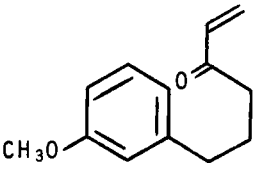
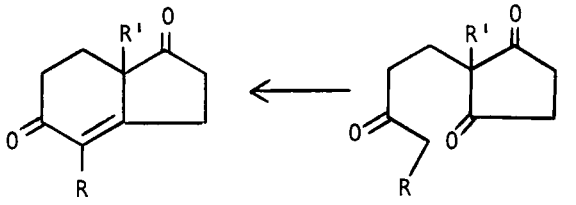


6

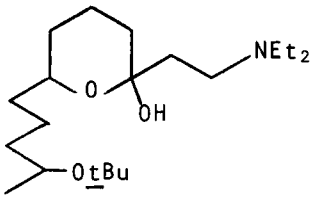
An interesting application of asymmetric synthesis afforded the indanedione 7 from the triketone 8 The use of aminoacids of the (S)-configuration such as L-proline and L-alanine as catalysts for the condensation gave optically active bicyclic intermediates 7 of the (S)-configuration in yields of 60-85% with optical purity in the same range.[4]



7 R = H, Me 8

R' = Me, Et

A total synthesis of norgestrel was described, in which the key step is the condensation of ethylcyclopentanedione with the tetrahydropyran derivative 9.[5] A similar method was used for the total synthesis of retrotestosterone and its derivatives.[6,7,8] A new method was devised for closing the D-ring, in which the acetylenic alcohol 10 is converted to the ketone 11 in 95% yield to give an 80:20 mixture of trans:cis ring junctures.[9]



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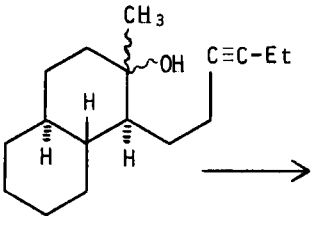

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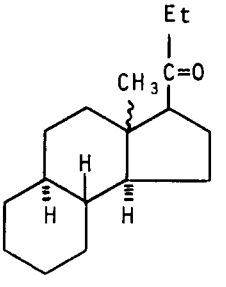


11

The application of the standard Torgov procedure for the preparation of 7,7-dimethyl-6-oxasteroids led surprisingly to the formation of the 8α,14β-isomer.[10] The authors postulate that a steric interaction between the gem-dimethyl group and the 15-proton caused isomerization of the Δ^{14}-intermediate to a 14β-Δ^{15}-isomer. Subsequent reduction of the Δ^{8}-double bond was reportedly influenced by the 14β-center to produce an 8α,9α-ring junction. However, when employing a similar synthesis to prepare a 7α-monomethyl-6-oxa system, the normal 8β,9α,14α-steroid was produced, although again some difficulty was encountered in the reduction of the Δ^{14}-bond to the 14α-isomer.[11]

The Torgov procedure was also used to prepare 2,4-dimethoxyestrone and the corresponding 3-desoxy derivative.[12,13] The former compound was of interest at one time because of reported analgesic activity.

A method for converting 19-nor intermediates such as 4 to 10β-methyl steroids was reported.[14] Epoxidation of the $\Delta^{5(10)}$-3-ketal 7 produced the 5α,10α-epoxide which was converted to the 10β-methyl-Δ^{4}-3-ketone by treatment with methyl magnesium bromide.

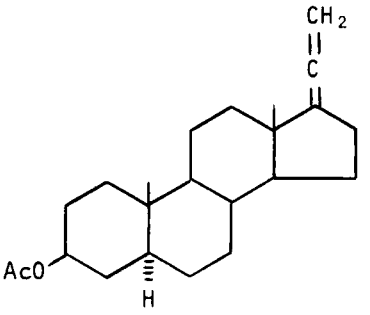
Other Synthetic Procedures - A new synthetic route, of possible commercial interest, to 6-dehydroretroprogesterone was reported. Progesterone, as either the 3,20-bisketal or the 3-monoketal, was converted to the corresponding $\Delta^{5,7}$-diene which was isomerized to the 9β,10α-isomer by photolysis. Hydrolysis afforded the desired 6-dehydroretroprogesterone.[15] Also formed in this reaction was the byproduct 6-dehydro-8α,10α-progesterone.

A new preparation of 19-norprogesterone was described in which diosgenin was converted to 19-nordiosgenin via the 6,19-oxide and the 19-methylol derivatives. Standard degradation of the side chain afforded 19-norpregnenolone in 30% overall yield.[16]

New methods for the synthesis of 17- and 17,21-hydroxylated side chains have been the subject of several investigations. Catalytic reduction of 16-methyl-16-dehydropregnenolone produced the 20-enol, which reacted with air to form the 17α-hydroperoxide. Reduction with triethylphosphite afforded 16β-methyl-17α-hydroxypregnenolone in 70% yield. However, attempts to prepare similarly the 16-unsubstituted analog resulted in much poorer yields.[17] Treatment with methyllithium of the 17β-cyano-17α-alcohol prepared from the 17-ketone afforded the 17α-hydroxypregnan-20-one system in good yield.[14] The latter could also be obtained from 17-vinylidine steroids (12), which are easily prepared from the corresponding 17β-acetoxy-17α-ethinyl steroids by treatment with zinc dust. Peracid oxidation of 12 yielded the 17α-hydroxy-20-ketone, whereas oxidation with osmium tetroxide afforded the 17α,21-dihydroxy-20-keto side chain in 53% yield.[18]

Some potentially useful new synthetic methods were reported. The addition of nitrile oxides to Δ^{16}-20-ketones, previously thought to give only 16α-carbon, 17α-oxygen-linked isoxazolines, has now been shown to produce also the isomeric 17α-carbon linked compounds (13).[19] If the

yields could be improved, these isoxazolines would be useful intermediates for the synthesis of progesterones containing novel 17α-carbon linked functionalized substituents.



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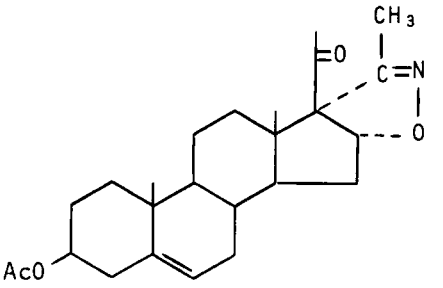


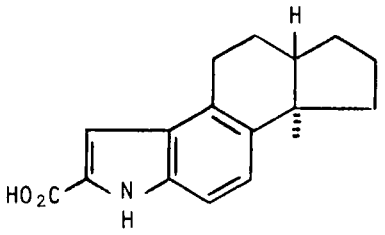
13

An innovative method for converting an aromatic A ring to a $\Delta^{4,9}$-3-keto system was reported,[20] a rather remarkable reversal of the usual aromatization process. The "remote photolytic oxidation" of steroids has now been extended to include oxidation at C_9 or C_{14} and C_{15} by attachment of the benzophenone reagent to 17β- or 24-alcohols.[21] The oxidation at C_9, which could lead to a variety of C-ring substituents including 11-oxygenated derivatives, appears to be a particularly valuable application of this new method.

The synthesis of A-nor steroids continues to be of interest, and several new routes have been devised. Pinacol rearrangements of the diols prepared by hydroxylation of 3-methyl-Δ^2-5α- and 3-methyl-Δ^3-5β-steroids led to 2-acetyl-A-nor-5α- and 3-acetyl-A-nor-5β-steroids respectively.[22] Formic acid-catalyzed rearrangement of a 1-methyl-1α,2α-epoxy-3-keto-androstane led in 70% yield to an epimeric mixture of 1-formyl-1-methyl-2-keto-A-norandrostanes, which was converted to the corresponding 1-methyl-2-ketone in good yield.[23] Other recent developments include the synthesis of 3α-methyl-2-keto-A-norandrostanes,[24] and the preparation of a 5α-methyl-3-keto-A-nor steroid by the pinacol rearrangement of 3α-methyl-3β, 5β-dihydroxy-A-norpregnan-20-one.[25]

Heterocyclic Steroids - Much synthetic work continues to be published in this area. Two non-basic aza steroids, 13-aza-17-ketones, were prepared with the stated purpose of testing a hypothesis that the usual lack of hormonal activity of azasteroids is due to an inability of basic steroids to penetrate cell membranes. However, no biological information was given.[26,27] Some 15-aza-,[28] 15,16-diaza-,[29] and 11,15,16-triazasteroids[30] were synthesized. The former two types are reported to have anti-bacterial activity, whereas 17a-aza-D-homosteroids are inactive.[31] The acetylation of one inactive 17β-hydroxy-4-azasteroid was shown to confer anti-bacterial activity to the compound, but this effect was not general.[32] The syntheses of a 5,10-diazasteroid[33] and of an A,B-indolosteroid (14)[34] were also reported. The successful preparations of 2-oxa,

2-thia, and 2-aza steroids were noted, but attempts to introduce a phosphorus atom into either the 2-[35] or 4-position[36] of the A-ring were unsuccessful. An interesting series of reactions of 2α,5α-oxido and 2α,5α-thio steroids led to a variety of A-nor-3-oxa and 3-thia steroids.[37] The synthesis of 5-azacholesterol[38] was reported, as well as further work on the syntheses of 8-,[39] 11-,[40,41] and 12-aza[42] systems. Preparations of the unusual structures, A-bisnor-B-homo-5-azapregnane-2,20-dione[43] and a series of 5α,7α-disulfides,[44] were announced.



14

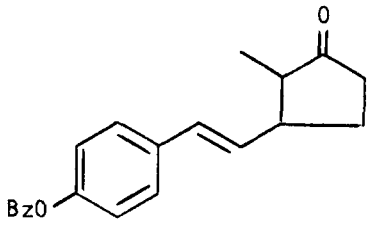
Miscellaneous Chemistry - Several other new steroid structures were prepared by a variety of synthetic routes. Among these are 8,19-oxido-steroids,[45] 9α-amino-11-oxygenated steroids,[46] a variety of 21-substituted-17-vinylidine steroids,[47,48] a 17β-,18-cyclosteroid,[49] and 9a-methyl-B(9a)-homo-A-norpregna-3(5),9a-diene-2,20-dione.[50]

Seco Steroids - The importance of the rigid steroid skeleton for hormonal activity has been fairly well established, but further investigations continue. The slight progestational activity of 15,16-secoprogesterone is not surprising, but the relatively high potency (ca. 1/5 x progesterone) of the isomeric 15,16-seco-17-isoprogesterone is rather unexpected.[51] Both 9,11-secoprogesterone[52a] and 5,6-secoprogesterone[52b] are inactive as progestins. Some weak androgenic activity was shown by 5,6-secotestosterone,[52b] but a 3,5-seco-5-keto derivative was inactive.[53]

A series of 9,11-seco-11-hydroxy and 11-carboxylic acid derivatives of equilenin were synthesized. It is noteworthy that of the four isomers tested, only that one which corresponds to the 13-epimer of the natural hormone has any significant estrogenic activity.[54] A very flexible B,C-bis-seco estrone derivative (15) was prepared and found to have only very weak estrogenic activity (ca. 1/250 x estrone).[55]

An interesting study of the importance of shape on estrogenic activity was accomplished by the synthesis of the two compounds (16, 17) in which the B and D rings of estradiol were "flipped over" to the "North" side of the molecule. Since 16 and 17 are very weak estrogens, and since they retain both the rigidity and the same accessability to the α- and β-faces as the normal estrogens, their low activity is most likely associated with changes in the "edge profile" of the whole molecule.[56] An intriguing analog of estrone (18) containing a spiro C,D-ring juncture was

prepared,[57] and 5,7-secomestranol was synthesized,[58] but no biological information was provided for either of these compounds.



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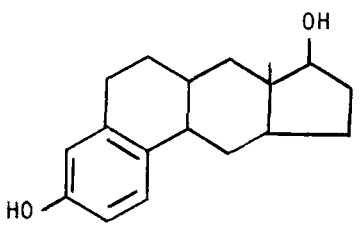

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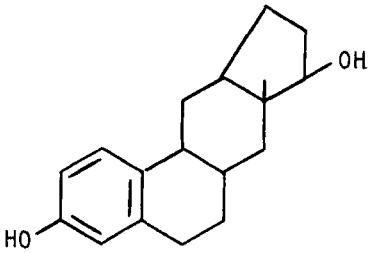

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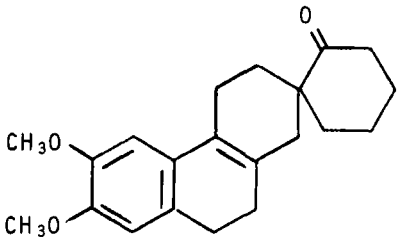
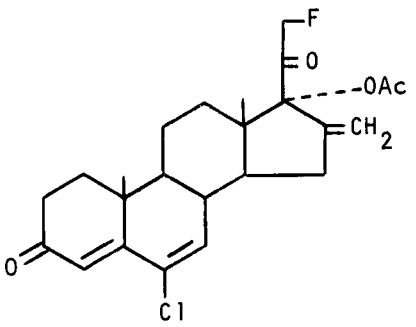


18

Progestins - Several very potent new progestational steroids were described. The 17α-propadienyl derivatives of norethindrone and norgestrel have oral progestational potencies 10 and 12 times, respectively, that of norethindrone.[59,60] The corresponding 17α-vinylethinyl derivative in the 19-methyl series also has gestagenic activity.[61] The 6,6-difluoro analog of norethindrone is two or three times more potent than norethindrone itself,[62,63] while the 18-vinyl analog of norethindrone is equipotent to norgestrel.[64]

The dihaloprogesterone derivative 19 is about 100 times more potent than progesterone,[65,66] whereas a variety of substituted 3-desoxy-17α-acetoxyprogesterones are all less active than the parent 17α-acetoxy-20-oxopregna-3,5-diene.[67] The introduction of a 4-chloro substituent enhances progestational activity,[68] but metabolism studies indicate the 4-chloro group is very labile, and, in fact, in vitro studies have demonstrated removal of this chlorine atom via nonenzymatic reaction with mercaptans. The proposed mechanism is discussed.[69,70] The introduction of several other substituents at the 4-position (e.g. cyano, formyl, aminomethylene) destroys hormonal activity.[71,72a,72b] The progestational activity of 17α-acetoxyprogesterone is enhanced five to ten times by the addition of a 7α-thioether moiety,[73] but hydroxyl substitution at C_7 reduces the usual potentiating effect of a 16-methylene group.[74]



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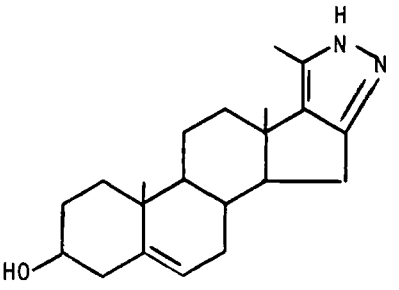


20

In a search for agents which would selectively alkylate the Clauberg "receptor," the synthesis and testing of 16α-substituted progesterone derivatives[75] and of ω-substituted esters of 17α-hydroxyprogesterone[76] was reported. Although several compounds displayed weak progestational activity, no evidence indicating receptor alkylation was obtained.

Estrogens and Androgens - In the estrogen series, the epimeric 17-carboxyestradiol-3-methyl ethers are reported and, interestingly, the "wrong" isomer, the 17β-carboxy-17α-estradiol ether is weakly estrogenic whereas the 17α-carboxy-17β-estradiol ether is inactive.[77] Thus there are three reported cases in 1971 (note the other two on p.186) of epimeric analogs of steroids in which that epimer corresponding to the less active natural steroid is the more potent analog. The reasons for this are unclear and obviously much remains to be learned about structure-activity relationships in the steroid field. In contrast to the progestin series, 18-methylestrogens are less active than the parent compounds.[78]

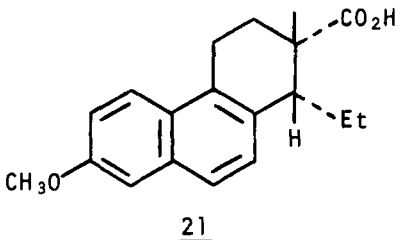
Further studies with 4-mercuri-17β-estradiol demonstrated that this compound effectively competes with β-estradiol for the 8S rat uterine cytosol estrogen receptor in an irreversible manner. Unexpectedly, the 8S-mercury steroid complex is not transferred to the particulate fraction under conditions in which the nuclear uptake of β-estradiol to a 4-5S complex could be demonstrated. Since the mercury steroid was previously shown to have biological actions typical of estrogens, the present observations are not in accord with current concepts that only intranuclear β-estradiol can trigger specific cellular responses.[79] Further evidence that the 4-mercury group undergoes mercaptide bond formation with the uterine estrogen receptor was obtained in studies with 4-mercuri-17α-estradiol. This compound has less estrogenic potency than the corresponding 17β-derivative and displayed persistent antiestrogenic activity equal in potency to 17α-estradiol but of much longer duration.[80]

In the androgen series, some 16α,17α-cyclopropano derivatives of 19-nortestosterone were synthesized in the hope that the smaller steric bulk of the methylene group, in comparison with that of a 17α-methyl group, would enhance oral androgenic activity; however, no biological data were

reported.[81] A 7-acetyl derivative of testosterone is being investigated as an antiandrogen, but again no biological data are available.[82] Some 8α-methyltestosterone derivatives were prepared but were nearly devoid of androgenic and anabolic activity.[83] Since 8α-testosterone retains most of the activity of the natural 8β-epimer, and since the intramolecular distances between the 3- and 17-oxygen atoms are about equal, the deactivating effect of the 8α-methyl group is rather unexpected, for it is currently believed that anabolic and androgenic agents approach their respective receptor sites from the β-side of the molecule.

Anti-fertility - The potential of steroids in the anti-fertility area is probably still the leading motivation for research in this field, but little new information has appeared. Two steroids, oxymetholone and nandrolone phenpropionate, both well established anabolic agents, were shown to cause resorption of fetuses after implantation. The term "interceptives" was proposed for this type of effect. This action of both compounds could be reversed by progesterone. It was postulated that oxymetholone suppresses pituitary gonadotrophin release, while nandrolone phenpropionate either inhibits luteal synthesis of progesterone or competitively blocks a uterine receptor site necessary for pregnancy maintenance.[84]

A pyrazoloandrostane derivative (20) has anti-implantation activity,[85] and 2α-aminodihydrotestosterone has moderate anti-uterotrophic activity.[86] Papers continue to appear on the anti-fertility activity of a variety of triarylethylene derivatives.[87-90] Extension of the post-coital anti-fertility effect of the methyl ester of dl-cis-bisdehydrodoisynolic acid (21) in monkeys to studies in humans would be of considerable interest.[91]



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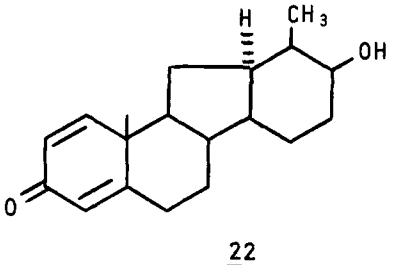


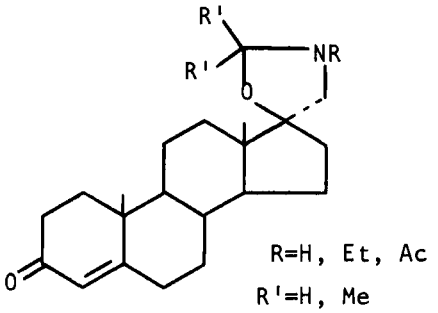
22

The clinical efficacy of diethylstilbestrol as a post-coital contraceptive in women was documented.[92] No pregnancies occurred in 1000 women given, within 72 hours of exposure, 25 mg of DES twice daily for five days. No serious side effects were reported and the majority of patients exhibited essentially normal menses following treatment. The use of DES as an emergency contraceptive is perhaps more tenable with the currently greater availability of backup abortion in case of failure.[93]

Miscellaneous Activity - In the corticoid area, several C_{21} derivatives of known active corticoids were prepared and evaluated.[94-98c] A new non-halogenated glucocorticoid possessing "extraordinary anti-inflammatory potency" was reported,[99] and the pharmacology of 9α-fluorohydrocortisone was extensively reviewed.[100] The relationships between structure and

biological activity and potency in the corticosteroid series were discussed.[101,102] Studies of the *in vitro* interaction between cortisone-21-iodoacetate and 20β-hydroxysteroid dehydrogenase led to the conclusion that the enzyme was irreversibly inactivated by alkylation of a histidine residue located at its active site.[103]

Two new types of steroids (22, 23) possessing anti-aldosterone activity were reported. A high level of activity was claimed for 22,[104] but 23 was only weakly active.[105]



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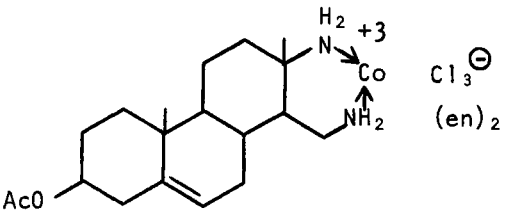

24

In the hypocholesterolemic area, 25-azalanosterol is superior to 20,25-diazacholesterol,[106] whereas the 17α-epimer of 20,25-diazacholesterol is inactive.[107] An unusual cobalt-containing diazasteroid (24) has a high degree of inhibition of cholesterol synthesis.[108] Several derivatives of 5α-cholestane-3β,5α,6β-triol were tested for hypocholesterolemic activity in rabbits.[109] None were as active as the parent compound. Members of this series do, however, inhibit the *in vitro* synthesis of cholesterol.[109,110]

Several 17β-aminosteroid derivatives were reported to have antimicrobial activity.[111] Others were found to rather specifically inhibit the biosynthesis of androgens from [21-^{14}C] 17α-hydroxyprogesterone by a rat testicular microsomal preparation. The structural analogy between the inhibitors and the natural substrate of the 17,20-lyase enzyme was pointed out, and it was postulated that the inhibitors resemble a natural enzymatic intermediate or transition state but, lacking a 17α-hydroxyl, cannot proceed to products.[112] The 10α-epimer of norethindrone has no progestational activity but is a strong estrogen.[113] Presumably this is due to a facile elimination of the 9,10-*cis* hydrogen atoms to form the aromatic A-ring.[114] Recent reports have stimulated renewed interest in the possibility of a medical treatment for gallstones. Chenodesoxycholic acid administration presumably reduces the "lithogenic potential" of bile by increasing the concentration of cholesterol-solubilizing components,[115] and further it has been reported to reduce the size of gallstones, as visualized by cholecystography, in 4 of 7 patients.[116,117] A mixture of 3α-hydroxy-5α-pregnane-11,20-dione and its 21-acetoxy derivative is reported to be a useful intravenous anesthetic.[118-120]

Considerable progress was made in elucidating the biotransformations of cholecalciferol (Vitamin D_3) to biologically active forms. The first of these has been identified as 25-hydroxy cholecalciferol, which in turn is further metabolized to the 1,25- and perhaps also to the 21,25-dihydroxy derivatives. Control over calcium transport is assured, since the initial hydroxylation is obligatory in conferring biological activity and the biosynthesis of the active metabolites is controlled by feedback regulation. While these hydroxylated derivatives, unlike Vitamin D_3 itself, are all directly effective at the cellular level, there is some evidence of organ specificity.[121,122]

Reviews - A number of review articles appeared, including two on the total synthesis of heterocyclic steroids.[123,124] Other reviews covered the search for catatoxic steroids,[125] retrosteroids,[126] and 16-substituted steroids.[127]

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Chapter 18. Peptide Hormones of the Hypothalamus and Pituitary.

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This survey will place emphasis on the more recent significant developments which have occurred during the two years since this subject was reviewed[1] with the aim of letting papers recently published in a given area lead the reader to earlier reports. Synthesis of hormones per se is covered in another survey (S.M. Stewart, this volume).

Hypothalamic Peptides.

Several general reviews and proceedings of conferences on hypothalamic peptide hormones have appeared since the last survey[2-6]. A comprehensive review on the chemistry of the hypothalamic releasing factors is forthcoming[7].

Methods of Bioassay - One of the difficulties in the past in the isolation and elucidation of the structure of the various hypothalamic releasing factors has been the problem of obtaining specific and reliable bioassays for these factors. The discussion of the limitations of the various bioassays is far beyond the scope of this survey and critical discussions can be found in some of the general reviews[2-7]. Radioimmunoassay of plasma levels of the various pituitary hormones has provided a more reliable endpoint for the determination of hypothalamic releasing factors. The use of dispersed pituitary cell cultures has greatly increased precision and quantitative ability of the releasing factor bioassay[8], as well as being more amenable to multiple treatment experiments than other assay methods. Another bioassay technique involves the use of radio-ligand binding to target cell receptor sites[9-10]. A radioimmunoassay for the direct determination of TRF has been described[11].

Thyroid Stimulating Hormone (TSH) Releasing Factor, TRF - The chemistry and physiology of TRF has recently been reviewed[3,12,13]. The structure of ovine TRF was conclusively established by high resolution mass spectrometry and additional chemical characterization to be $\square$Glu-His-Pro-NH_2[14]. Subsequently, porcine TRF was shown conclusively to have the same structure[15,16].

The first chemical synthesis of TRF involved the ammonolysis of the methyl ester prepared from the free acid, $\square$Glu-His-Pro-OH[17]. Subsequently, many different kinds of total syntheses of TRF have appeared, employing classical[18-20] and solid-phase methods[19,21,22] using chloromethyl resin or a benzhydrylamine resin[12]. The latter method was used to synthesize TRF labelled as [^{3}H]-Pro (ca. 50 Ci/mM)[7].

Well over 50 structural analogues of TRF have now been synthesized and tested for biological activity. Many of the results of these studies have been tablulated and discussed elsewhere[7,12,13]. Most of the modifications, which have included the alteration or replacement of every amino acid, have resulted in substantial reduction of the biological activity,

indicating that every part of the molecule is an essential requirement for biological activity. Substitution of the histidine by more basic amino acids such as arginine, lysine[23,24], α,γ-diaminobutyric acid[23], or ornithine[24], yields compounds with little activity. L-Glu-His-(πMe)-Pro-NH_2 has .04% of the TRF biological activity, but L-Glu-His(τMe)-Pro-NH_2 has 800% activity[24]. Interestingly, the dipeptide L-Glu-His(τMe)-NH_2 exhibits some biological activity[24]. It is unclear whether or not the biological effects observed result from inductive effects of the methyl group on the aromatic ring or are due to steric effects. If the 2-position of TRF is substituted by Phe[25], or by β-(pyrazolyl-3)-alanine[23,26], a considerable amount of biological activity is retained; apparently, the aromaticity of the imidazole ring is important. L-Glu-Tyr-Pro-NH_2[Tyr^2]TRF, has much less activity than [Phe^2]TRF, probably because of the influence of the hydroxyl group[25]. The stereoisomer "L-D-L"-TRF (L-L-Glu-D-His-L-Pro-NH_2) exhibited 3% of the biological potency of the native compound, the "L-L-D" and "D-L-L" analogues were much less active, and "D-D-D"-TRF was inactive at the concentrations studied[27]. No structural analogues of TRF have been reported to act as inhibitors.

The binding of TRF to whole pituitary cells in culture or plasma cell membrane fractions in competition with [^{3}H]TRF has been studied[7,9,10,28]. The binding affinities of the TRF analogues have shown a close correlation with the observed biological activity, suggesting that the limiting factor in biological activity is the affinity for the receptor.

Initial studies on the biosynthesis of TRF indicate that it may be non-ribosomal[29-32]. TRF is rapidly inactivated *in vitro* by plasma, serum or whole blood. The evidence best supports enzymatic inactivation[33,34]. One of the major products appears to be the free acid, L-Glu-His-Pro-OH[33]. The dipeptide L-Glu-His-OMe, which has little or no TRF activity, apparently acts as a competitive inhibitor of plasma inactivation of TRF[34].

The clinical use of TRF has been reviewed[11,35]. Synthetic TRF has been extensively tested in patients to test pituitary TSH reserve or pituitary response to TRF. Doses have ranged from 250 μg to 1 mg i.v. and 2 to 10 mg orally with no serious adverse effects.

TRF has been shown to be highly specific in the release of TSH, having no effect on the secretion of luteinizing hormone (LH), follicle stimulating hormone (FSH), adrenocorticotropic hormone (ACTH) *in vivo* or *in vitro*[3,5]. Recently, Tashjian *et al.* have reported that TRF stimulates the release of prolactin (PRL) *in vitro*[36]. These results have been confirmed by other laboratories[7]. As in the case with TSH, the effect of TRF on the release of prolactin is inhibited by thyroxin. Although large amounts of TRF are required to achieve an effect in rats, it appears to be relatively more potent for the release of prolactin in man[37,38].

Luteinizing Hormone Releasing Factor (LRF) and Follicle Stimulating Hormone Releasing Factor (FRF) - Porcine[39] and ovine[40] LRF have now been identified as having the same primary structure, L-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2. A review of the work on the porcine material by

Schally's group has appeared[41]. The results from Folkers' laboratory on partial characterization of bovine and porcine LRF have been reviewed by Sievertsson[13].

The first description of the total synthesis of the LRF decapeptide was that of Monahan et al.[42]. The peptide had the biological activity of ovine LRF in vivo and in vitro (rats) and caused ovulation in rabbits[42,43]. Subsequent to that report, several other descriptions of syntheses have appeared which employed classical methods[13,44] or solid-phase methods[13,45,46].

Judging from programs of forthcoming meetings and conferences in June, 1972 (e.g., IVth Intern. Congr. of Endocrinology, Washington; Serono Fdn. Conf., Acapulco, Mexico), there is a considerable amount of activity now in the area of synthesis and biological testing of analogues of LRF. However, at this writing, only a small portion of this has appeared in print. In our own laboratory[7,47], we have tested, among others, synthetic LRF peptide amides shortened from the C-terminus (i.e., pGlu-NH_2 through des-Gly^{10}-LRF). The nonapeptide des-Gly^{10}-LRF has about 10% of the potency of LRF in the pituitary cell culture assay, and the other shorter peptides, with the exception of pGlu-His-Trp-NH_2 which has a .13% activity, have little or no activity, ($\leq$0.02%). It is interesting, however, that some of the shortened peptides, including pGlu-His-NH_2, have some intrinsic biological activity. The greater activity of the tripeptide suggests that the C-terminal portions of the larger peptide analogues may be actually interfering with the biological activity of the tripeptide portion of the molecule. Folkers' laboratory[13], in collaboration with Bowers, has tested several di- and tripeptide analogues of LRF. None of these, including pGlu-His-NH_2, (see below) were found by them to have LRF activity at the doses tested. In a series of 6 tetrapeptide-amides having a pGlu N-terminus in combination with Tyr, Arg, and Trp, only one, pGlu-Tyr-Arg-Trp-NH_2, was reported to have some LH releasing activity[13]. In our hands, the tetrapeptide pGlu-Tyr-Arg-Trp-NH_2 has less than .001% of the activity of LRF, which is less than we find for the dipeptide pGlu-His-NH_2 (.02%)[7,47]. The discrepancies in the observations of the two laboratories[7,13,47] regarding the ratios of LRF activity of pGlu-His-NH_2 and pGlu-Tyr-Arg-Trp-NH_2 may not be significant since the levels of biological activity are extremely low and the methods of bioassay are difficult to compare. A tetrapeptide, pGlu-His-Pro-Gly-NH_2, which is 35% active as a TRF, also has distinct LRF activity indicating that the receptors, although not identical, might have some degree of similarity[7]. We have also tested[7] a series of analogues in which substitutions in the various amino acid residues have been made. Placing the methyl group on the π- or τ-imidazole position of histidine results in analogues with 3% and 6% LRF activity, respectively. These effects are in contrast to those observed for TRF in which the τ-methyl substitution greatly enhances biological activity. In a series of analogues in which glycine has replaced each of the various amino acids, all of the analogues show considerably reduced biological activity. In the in vitro cell culture system, one of these analogues, [Gly^2]LRF, in addition to a slight agonist activity, showed competitive inhibition of LRF activity. The nonapeptide analogue in which histidine had been deleted, des-His^2-LRF, showed very little activity as an agonist and was also able to antagonize

competitively the action of LRF[48,49].

Claims have appeared in the literature of the separation from hypothalamic extracts of a separate FRF from LRF activity, but these conclusions have been disputed on the basis of the techniques of bioassay[2,3,5]. It is now well established that the natural product isolated as LRF also possesses considerable FSH releasing activity in all the systems tested[7,41]. Furthermore, the synthetic LRF decapeptide, like the natural product from porcine and ovine material, releases FSH. The analogues of LRF which we have tested so far also have FSH releasing activity in about the same proportion, but the FSH releasing potency is much less than that of LH and, although it is statistically significant, it is much more difficult to quantitate. In contrast to the claim of Sievertsson et al.[13], that the tetrapeptide □Glu-Tyr-Arg-Trp-NH_2 is inactive as an FRF at high doses, we have found it to have about the same ratio of FRF/LRF activity as other analogues tested[7]. Although the LRF decapeptide is capable of releasing both LH and FSH, the question of whether or not there exists a separate chemical entity which releases FSH, remains to be established. Porcine[41], ovine[50] and synthetic LRF[41,51], stimulate the release of LH and FSH in human beings. Discussions of possible clinical use of LRF in fertility control have appeared[41,52,53].

Growth Hormone (Somatotropin) Releasing Factor, SRF (GRF) - Considerable evidence from physiological and clinical areas strongly supports the existence of hypothalamic control of growth hormone release[2-6,54]. The purification of hypothalamic factors influencing the content of pituitary growth hormone has been reported by several laboratories, but the significance of assays based on pituitary hormone content has been questioned[54]. Schally et al.[55] have reported the purification and sequencing of the peptide Val-His-Leu-Ser-Ala-Glu-Glu-Lys-Glu-Ala from porcine hypothalami on the basis of this assay, and Veber et al.[56] have synthesized the peptide. Both the native peptide and its synthetic replicate were active in the pituitary depletion assay but failed to release radioimmunoassayable growth hormone in the plasma of normal animals or humans. Moreover, Veber et al.[56] have noted the structural similarity of "GRH" to that of the amino terminal sequence of the β-chain of porcine hemoglobin. The physiological significance of "GRH" thus remains to be established. Several laboratories[57-59] have reported that extracts of hypothalami (porcine, ovine, rat) and partially purified materials from these extracts stimulate release of radioimmunoassayable growth hormone. Others have reported growth hormone release inhibiting activity in crude hypothalamic extracts[60,61]. The chemical nature of any of these substances has not been characterized.

Corticotropin Releasing Factor, CRF - Although CRF was one of the first of the releasing factors to be studied, the chemical nature of this factor still remains elusive, partly because of the difficulties in the bioassay techniques[2-7] and possibly also because of chemical instability of this hormone[62]. It is now the consensus of most of those working in the field that a hypothalamic CRF does exist and that although vasopressin is capable of releasing ACTH in the various assay systems, it is probably not the physiological mediator of ACTH release[2-7]. The exact relationship of hypotha-

lamic CRF to either vasopressin or α-MSH or both, as well as its relationship to CRF of neurohypophysial origin, remains to be established[3,5]. Chan et al.[62] have suggested that there may be two CRF's in the rat hypothalamus.

Prolactin Release Inhibiting Factor, PIF, and Prolactin Releasing Factor, PRF - There is physiological evidence that the secretion of prolactin is under some sort of tonic inhibition by the hypothalamus[2-7]. Descriptions of attempts to purify this factor have placed it in or near the zone of material having LRF releasing activity in gel filtration experiments. LRF does not stimulate or inhibit the release of prolactin in vivo or in vitro [63]. No meaningful chemistry on the nature of this factor is available.

The ability of TRF, (Glu-His-Pro-NH_2, to release prolactin has already been mentioned. The realtionship of TRF to a possible natural PRF in hypothalamic tissue remains to be established.

Melanocyte Stimulating Hormone (Release) Inhibiting Factor, MIF, and Releasing Factor, MRF - Like prolactin, the release of MSH appears to be under a tonic inhibition by the hypothalamus[2-7]. Celis et al[64] have proposed that the C-terminal fragment of oxytocin, Pro-Leu-Gly-NH_2, is an MSH release inhibiting hormone, MIF; this suggestion was confirmed by Nair et al[65], who sequenced material obtained from the hypothalamus. Both groups supported their observations using synthetic material. The MIF activity of this peptide could not be confirmed by other laboratories[7,66]. On the other hand, the ring structure of oxytocin (tocinoic acid), Cys-Tyr-Ile-Gln-Asn-Cys-OH, was reported to exhibit some MIF activity[66]. Both of these peptides may arise from enzymatic degradation of oxytocin in the hypothalamus[64]. The pentapeptide Cys-Tyr-Ile-Gln-Asn-OH has been reported to exhibit MSH releasing activity[67]. These results remain to be confirmed. The significance of these peptides as mediators of MSH secretion remains to be established in view of the questions which arise concerning the validity of the assay systems.

Mechanism of Action of Hypothalamic Releasing Factors - The mechanisms of action of hypothalamic factors have been reviewed[5,6,12,68]. Effects of $[K^+]$, $[Ca^+]$, other cations and metabolic inhibitors, as well as possible roles of cyclic-AMP and prostaglandins in the release of pituitary hormones are discussed. The acute effects of release of TSH and LH by TRF and LRF respectively appear not to be affected by pretreatment with inhibitors of protein synthesis, such as cycloheximide, puromycin or actinomycin D, but conflicting reports occur regarding the effect of these inhibitors on the release of other pituitary hormones. The acute or chronic effect of the releasing factors on the synthesis of pituitary hormones has been studied using crude hypothalamic extracts and measuring levels of the pituitary hormone in various tissues and fluids. The effects observed are minimal with respect to the confidence limits of the bioassays involved; studies using highly reliable measurements of the pituitary hormones are quite limited. ^{14}C-glucosamine and ^{14}C-alanine had been reported to be incorporated into immunologically precipitable thyrotropin. A similar report of the incorporation of ^{3}H-glucosamine into LH by highly purified porcine

LRF has recently appeared[69].

Pituitary Hormones.

The technique of radioimmunoassay of pituitary hormones is now well established[1,70]. Assays of human anterior pituitary hormones as tests of pituitary function have been reviewed[71]. Reports have appeared describing radio-ligand binding of pituitary hormones to target tissues for mechanistic studies or as a possible means of assay[72,73]. The biological action of ACTH[74] and bovine growth hormone (BGH)[75] using hormones bound to large molecules, has also been studied.

Growth Hormone - The structure of human growth hormone (HGH) as originally proposed by Li et al[76], has been revised[77,78]. The pentadecapeptide fragment containing Trp originally assigned to position 17-31 was found to occupy positions 77-91; a missing dipeptide fragment, Leu-Arg, was found in positions 92-93 and the sequence Gly-Ser-Pro in 130-132 was corrected to Pro-Ser-Gly[77,78]. In addition, the Asp residue at position 47 and Glu at positions 49,90 and 121, were reassigned as Asn and Gln, respectively[78]. A synthetic preparation of a peptide obtained by solid-phase methods based on the originally proposed growth hormone sequence had 10% growth promoting potency and 5% lactogenic activity in comparison to the native hormone, and it also reacted immunologically with rat antiserum to HGH[79]. Li et al[80,81] have also reported the synthesized protected peptide fragments by classical methods based on the original structure. Chillemi and Pecile[82] have reported that peptides corresponding to the sequence 81 to 121 and the sequence 122 to 153 of HGH[76] prepared by solid-phase synthesis, were active in the tibia test. As noted above, the sequence upon which these syntheses were based has been revised considerably in the 81-121 region, although the sequence of 30 of the 41 amino acid residues are in segments homologous to the revised sequence. It is, nevertheless, of interest that such small segments of the GH molecule would have activity. These conclusions are in agreement with earlier reports that growth hormone fragments obtained from proteolytic or cyanogen bromide cleavage retain some biological activity[82]. Reports from various laboratories have appeared on sequence studies of growth hormones of other species. Fellows et al.[83] have provided evidence that the multiple chains of BGH arise from enzymatic degradation of the N-terminal portion. Differences may occur, however, in addition to the N-terminal sequences[84,85]. Sequence studies show that BGH[86-88], ovine GH[89,90], and porcine GH[91], have a high degree of homology with HGH, especially in the C-terminal position.

Prolactin, PRL - Bovine prolactin (BPRL) has been reported[92] to differ from the sequence of ovine prolactin (OPRL)[93] by only two amino acid residues. The prolactins show considerable structural homology with growth hormone and with human placental lactogen (more recently called human chorionic somatotropin, HCS), HCS shows a high degree of homology with GH; over 80% of the amino acids are identical and the remaining pairs are related by highly favored codon substitutions[77,94]. Nicoll and Licht[95] have compared the elctrophoretic mobilities of prolactins and somatotropins from a variety of species and have reviewed extensively the functional

relatedness of these molecules. In all the tetrapod species examined, prolactins and growth hormones appeared to be separate molecular entities but both hormones shared prolactin and somatotropic activities. Niall et al.[77] have observed that, in addition to the "external homologies" of prolactins and growth hormones, a considerable degree of "internal homology" exists, there appearing to be four regions in which sequences of approximately 20 amino acids are clearly related within the individual molecules. These findings had led them to suggest that these hormones have all arisen from a shorter primordial peptide by gene reduplication. Evidence is mounting, primarily on the basis of immunological studies, that a human prolactin separate from human growth hormone, exists[96-98]. Continued reports have appeared concerning the physical-chemical properties of HGH[99-101], BGH, OGH[102], and OPRL[100].

Glycoprotein Hormones; Thyroid Stimulating Hormone, TSH, Luteinizing Hormone, LH, Follicle Stimulating Hormone, FSH - The complete primary structures of bovine TSH[103] and ovine LH[104-106] have now been reported. The structural studies on TSH and comparisons of TSH with other glycoprotein hormones have been reviewed by Pierce[107]. These two hormones[103-107] as well as FSH[108-111], dissociate into two separate chains of approximately 15,000 molecular weight each. The hormones contain about 15-30% carbohydrate, although the sugar contents vary. Bovine and ovine TSH and LH contain little or no sialic acid whereas human TSH and LH and FSH are reported to contain more sialic acid[107] which apparently is necessary for full hormonal activity[112,113]. However, desialylation of human LH increases binding affinity to subcellular testis or ovarian fractions[114] and also seems to enhance immunologic activity[115]. The structure work on TSH and LH revealed that one subunit, TSH-α and LH-α, is essentially the same in both hormones and the other subunits, TSH-β and LH-β, are the hormone specific units; not only can the α and β subunits of a given hormone be recombined to give a reconstituted complex with the chemical and biological properties of the parent hormone, but also either TSH-α or LH-α can be combined with TSH-β to give TSH and either α-chain can be combined with LH-β to give a molecule with the properties of LH. A similar relationship exists between the subunits of human chorionic gonadotropin (HCG) and the subunits of TSH and LH. The α subunits of human LH, FSH and human HCG[116, 117] can also be substituted for each other in combination with the β subunit of the other hormones[116]. The sequence of the ovine LH-β subunit reported by Ward's laboratory[105] and bovine LH-β[118] are now in complete agreement; the sequence proposed by Li's group[106] for ovine LH-β differs from these proposals by the placement of 17 amino acids, most of which represent reversal sequences. Also, Ward et al.[105] propose an acylated amino terminus in contrast to the findings of Papkoff et al.[106]. There is a striking degree of homology (around 50%) between the TSH-β and LH-β sequences[107]. Hennen et al.[119] have noted an "internal homology" in the α-chain of bovine LH in a situation analogous to that observed by Niall et al.[77] for growth hormone and the lactogens. Chemical and biological characterization of the glycoprotein subunits is continuing. There appears to be agreement that the circular dichroism (CD) of intact ovine LH indicates little or no helical character but there is some disagreement regarding the CD character of the subunits[120-122]. Yang et al.[123] report that the

LH-β subunit induces ovulation in the hamster. The ovine LH-α subunit has lipolytic activity as does the intact hormone, whereas the LH-β is inactive to release free fatty acids from mouse adipose tissue[124]. The β-subunit appears to provide most of the antigenicity of ovine TSH[107] and LH[107,124-126].

Adrenocorticotropic Hormone, ACTH - Reports on biological activities of synthetic ACTH and its analogues are continuing. Fragments as small as $ACTH_{4-10}$ (porcine corticotropin (4-10) heptapeptide) and $ACTH_{5-10}$ hexapeptide exhibit steroidogenic activity[127]. $ACTH_{11-24}$ is a competitive antagonist of $ACTH_{1-39}$ (porcine), suggesting that the region 11-24 is not involved in activation but provides affinity for the receptor[128]. $ACTH_{1-24}$ diazotized to polyacrylamide stimulates steroidogenesis in rat adrenal cells without entering the cell, suggesting that there is a possible interaction of the ACTH at the cell surface receptors[129]. ACTH diazotized to agarose induced steroidogenesis without entering the cell but, apparently did not adhere to the cell surface[130].

Reports from several laboratories have now appeared, implicating the involvement of ACTH or ACTH analogues in memory or behavioral responses[131-133,135]. A peptide "BC" has been obtained from pituitary which stimulates avoidance acquisition in rats but has no ACTH, MSH or pressor activity[134]. The zinc phosphate vehicle used in some of these studies is also able to mimic some of the behavioral effects in the conditioned avoidance response[135].

Proton NMR studies have led Patel[136] to conclude that no interaction occurs between sequences 1-10 and 11-24 in $ACTH_{1-24}$. Structural relationship of ACTH to biological activity has been discussed by Toniolo[101].

Melanocyte Stimulating Hormone, MSH - Shapiro et al.[137] have studied MSH activities in a variety of vertebrate species, comparing these to known α- and β-MSH's, and have concluded on the basis of gel filtration behavior, radioimmunlogical assays and bioassays that the structures of the major MSH hormones in several of the species are different from those which have been characterized previously. Sandman et al.[138] have claimed involvement of MSH in the passive avoidance response.

Lipotropic Hormone, LPH - The involvement of pituitary hormones in releasing free fatty acids from adipose tissue (lipolysis) has been discussed at a symposium[139]. The amino acid sequence (58 amino acids) of porcine α-lipotropic hormone (α-LPH) is closely homologous to that previously reported for ovine γ-LPH. The porcine γ-LPH sequence appears to be identical to the N-terminal 1-58 sequence of porcine β-LPH in a relationship similar to that previously observed for β- and α-LPH of bovine origin. Also, as has been the case with the ovine hormone, the C-terminal sequence of porcine γ-LPH is identical to the entire 18 amino acid sequence of β-MSH from the same species. Partial characterization of human lipotropins was described.

Posterior Pituitary Hormones; Oxytocin, Vasopressin - The chemistry, physiology, and assay of oxytocin and vasopressin have been extensively reviewed[140]. Investigation of the properties of synthetic neurohypophysial hormones continues to be a very active area. [Thr^4]oxytocin exhibits enhanced oxytocin-like activities and diminished vasopressin-like activities; [Thr^4]mesotocin exhibits similar properties[141]. Recent studies[142] have revealed the somewhat surprising result that removal of the N-terminal amino group from these derivatives results in reduction of biological activity rather than enhancement as might have been expected in the light of previous observations concerning oxytocin and other 4-substituted analogues of oxytocin[143]. Manning et al.[142] have suggested that the solubility differences of these various analogues offer one possible explanation for the differences in biological properties. The laboratory of du Vigneaud has described a series of analogues of oxytocin having the Cys residue in position 1 replaced with L-penicillamine (L-β-β-dimethylcysteine) derivatives which are devoid of avian vasodepressor, oxytocic, and pressor activities but which inhibit the oxytocic activity of oxytocin[144]. [Leu^2, Leu^4]oxytocin, which has natriuretic activity and weak diuretic effects, is also an inhibitor of oxytocic activity[145] in contrast to [Leu^4]oxytocin which has considerable diuretic and natriuretic effects as well as anti-ADH activity (inhibiting the antidiuretic effect of vasopressin); other 4-substituted oxytocins have been reported to have anti-ADH effects[146]. Conformations of oxytocin, vasopressin and their derivatives have been studied by NMR[147-149]. The sequence of porcine neurophysin I has been determined [150].

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Chapter 19. Non-steroidal Antiinflammatory Agents

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Introduction - Research in the field of non-steroidal antiinflammatory agents (NAA) encompasses too wide an area to be completely covered in a review of this size. As was the case in last year's Report,[1] we have had to confine our survey mainly to the work relating to the chronic arthritic diseases. For those readers interested in the broader aspects of the subject, several excellent texts[2,3] and reviews[4,5] are now available. Reports have appeared on three important symposia.[6-8]

Etiology and Pathogenesis - The widely held belief that infectious organisms play some part in the initiation of rheumatoid arthritis (RA) is proving difficult to substantiate. New investigations[9,10] have indicated that mycoplasma may be implicated in the pathogenesis of RA, and skeletal lesions have appeared in mice[11] and chicks[12] which have received injections of human rheumatoid synovial tissue. A recent study,[13] however, has demonstrated no significant benefit from long term tetracycline therapy for patients with RA.

Animal Test Models - In vitro screens for NAA have been largely neglected, with continuing reliance being placed mainly on the adjuvant arthritis and carrageenin foot edema models in the rat. By the use of both non-established and established adjuvant diseases it has been possible to differentiate between immunosuppressive agents and purely antiinflammatory agents.[14,15] Di Rosa and Willoughby have delineated[16] the sequence of events in the carrageenin-induced foot edema model. The initial mediators, histamine, serotonin, and kinins, are followed by a prostaglandin, the release of which is associated with the migration of leukocytes into the inflamed site. NAA suppress this migration. Experimental rabbit models of osteoarthritis have been reported.[17,18]

Modes of Action - To add to the many existing proposed modes of action of NAA, several new hypotheses have been advanced. It has been suggested[19-22] that acidic NAA owe their effectiveness to their demonstrated ability to inhibit the synthesis or release of prostaglandins such as PGE_2 and $PGF_{2\alpha}$. The prostaglandin blockade occurs at drug concentrations well within the range attained clinically in plasma.[19] Prostaglandins have been implicated as mediators in certain types of inflammatory conditions in the rat[16,23-26] and in man,[27] but their role in RA has not been established. Perhaps the prime action of the NAA is to inhibit the release of damaging lysosomal enzymes from invading leukocytes, since these enzymes are envisioned[28] as being responsible for the ultimate production of the prostaglandins.

It has been proposed[29] that antirheumatic drugs act by displacing certain small peptides from their binding sites on serum proteins. These free peptides can then protect connective tissues against the effects of

chronic inflammatory reactions. Patients with RA have a relative deficiency of free peptides.

Several acidic NAA have been shown[30] to inhibit nicotinate phosphoribosyl transferase in human platelet lysate by reversible competition with nicotinic acid. The resulting suppression of nicotinamide adenine dinucleotide biosynthesis may inhibit mucopolysaccharide biosynthesis and reduce the inflammatory response.

Still a matter of some controversy is whether NAA either stablize or labilize lysosomes. The answer seems to depend on the source of the lysosomes and the experimental conditions used in the assay.[31,32]

Shock - Considerable interest has been shown of late in the use of NAA against shock due to various causes. Treatment of dogs with NAA abolished some of the symptoms of shock caused by injection of living E. coli organisms.[33] Doses of NAA required for activity against cholera toxin in the rat were in the same range as those required for antiinflammatory activity.[34] Membrane stabilization effects[34] and the inhibition of prostaglandin release or synthesis[35] have been suggested as possible modes of action for the drugs, although a brief report[36] has appeared on the beneficial effect of PGE_1 and $PGF_{2\alpha}$ in endotoxin shock.

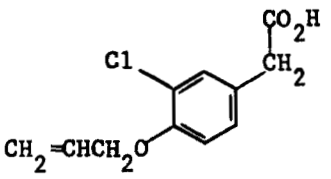
Agents under Investigation *

General - A working draft of clinical testing guidelines for NAA has been introduced by the FDA.[37] Of particular interest is the proposal that compounds are needed with less side effects than aspirin without necessarily any greater effectiveness than aspirin. An excellent survey of the problems associated with the clinical assessment of drugs for use against RA has been published.[38]

Arylalkanoic Acids and Related Compounds - Without doubt this area has been the most widely explored for potential NAA. In a double blind crossover trial, 1.5 g/day of alclofenac (1) was judged equivalent to 75 mg/day of indomethacin in patients with osteoarthritis of the hip.[39] In a separate study, 3.0 g/day of 1 was effective in 60% of patients with RA, ankylosing spondylitis, and osteoarthritis.[40] Clinical improvement with the absence of side effects was noted in rheumatoid arthritics receiving the ethanolamine salt of 1 at 0.5-1.5 g/day, i.m.[41] About 70% of 983 patients with RA, osteoarthritis, and allied conditions have shown improvement on long-term therapy with ibuprofen (2).[42] In a review on 2, however, it was concluded that 2 was an effective analgetic agent but has not so far been shown to have antiinflammatory (AI) effects in man.[43] Fenoprofen (3), an antiinflammatory-analgetic agent, was reported to inhibit swelling in the carrageenin rat foot edema test (CE), inhibit adjuvant arthritis, and to have an ED_{50} of 0.5-1.0 mg/kg in the UV erythema assay.[44] The (+)- and (-)-isomers appeared equipotent. The major urinary metabolites of 3 in the rat and rabbit were free and conjugated 4'-hydroxy derivatives.[45] Patients

* Unless stated otherwise, agents were administered orally.

with RA who were treated with 3 obtained symptomatic relief after 4 days.[46]



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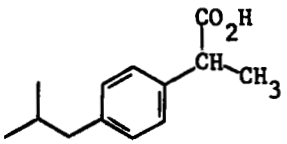

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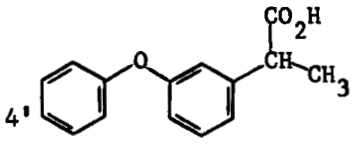
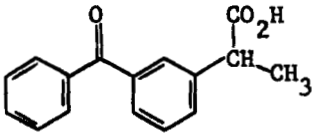


3, (±)

Activity comparable to indomethacin in the carrageenin abscess assay in the rat was reported for 4 (19 583 R.P.) which is apparently undergoing clinical evaluation.[47] A comprehensive pharmacological paper on the AI and analgetic properties of naproxen (5) has appeared,[48] together with a claim that the compound significantly reduced hind paw inflammation in the established adjuvant arthritis assay after 14 daily doses of 0.2 mg/rat.[49] A clinical trial has indicated 5 to be effective against RA at doses of 300-500 mg/day.[50] Tolmetin (6) had 0.38 times the potency of indomethacin (CE) and was claimed to be active in the adjuvant arthritis assay.[51]



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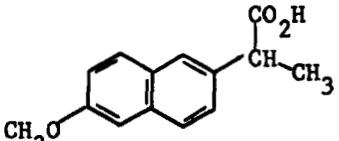

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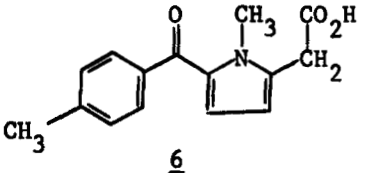
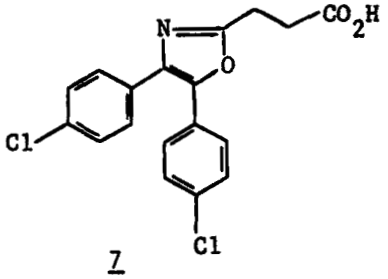


6

Wy 23205 (7) had twice the potency (CE) of phenylbutazone and was slightly less potent than indomethacin in the adjuvant arthritis assay.[52] Leo-1028 (8) had a minimal effective dose of 13 mg/kg (CE).[53] Comparable potency to indomethacin was claimed for the racemic acid 9 (TAI-284) in a wide variety of acute and chronic AI animal screens.[54] Most of the activity resided in the (S)-(+)-isomer.[55] Evidence has been presented[56] which does not support the proposal[55] that the AI activity of 5-cyclohexylindan-1-carboxylic acids is due to a steroid-like mechanism.



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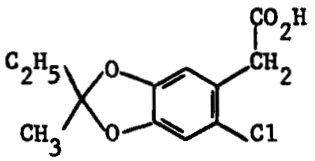

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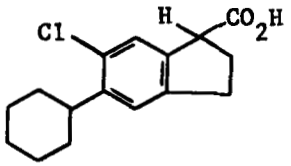
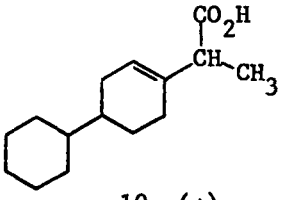


9, (±)

Compound 10, with the usual aromatic ring partly saturated, had only 0.05 times the potency of indomethacin in the adjuvant arthritis assay.[57] Compound 11 was equipotent to indomethacin (CE), with an LD_{50} in rats of between 50 and 136 mg/kg.[58] Metiazinic acid (12) had a prominent analgetic effect at daily doses of 0.75-1.5 grams in patients with various rheumatic

afflictions.[59]



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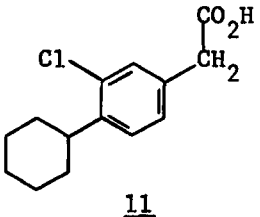

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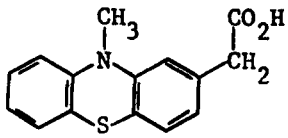

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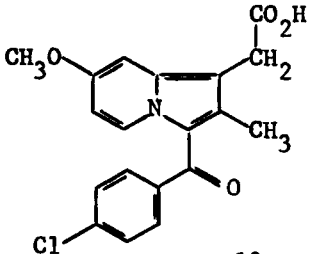
The indolizine isomers 13 and 14 had about 0.2 times the potency (CE) of indomethacin (15).[60]



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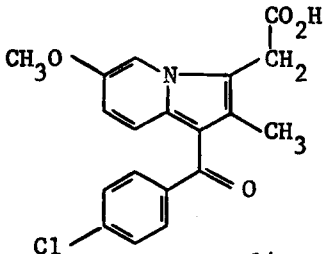

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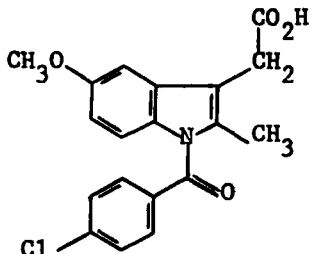
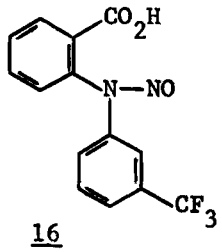


15

Fenamic Acids and Aza Analogs - The N-nitroso derivative 16 (ITF 611) was reported to have twice the potency (CE) of flufenamic acid, with less toxicity.[61,62] Clonixin (17) had 0.5 times the potency (CE) of flufenamic acid,[63] and in a clinical trial 600 mg of 17 was found comparable to 12 mg of morphine sulfate (i.m.) in the relief of postsurgical pain.[64] At daily doses of 0.75-1.0 g, niflumic acid (18) was shown to be of some benefit in a wide variety of rheumatic afflictions.[65]



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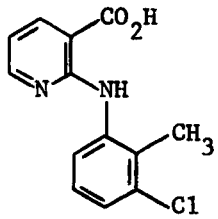

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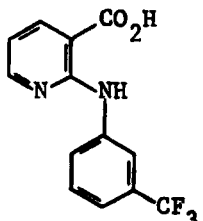
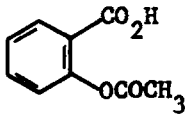


18

Salicylic Acids - Three days after substitution of aspirin (19, 3.6 g/day) by placebo, exacerbations were evident in approximately two thirds of patients with RA.[66] Benorylate (20) at 4 g/day[67] was found to be as effective as aspirin (3 g/day) in RA, and at 6 g/day[68] lowered the erythrocyte sedimentation rate more significantly than phenylbutazone (0.32 g/day), although the patients preferred the phenylbutazone. Flufenisal (21) may not possess significant advantages over aspirin to justify continued clinical evaluation.[69]



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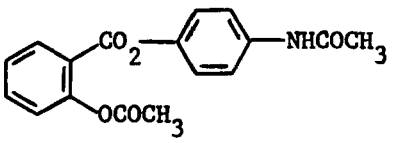

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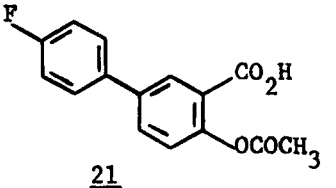
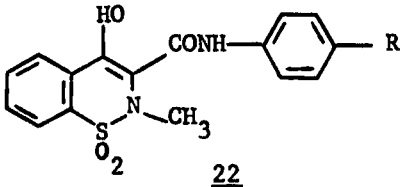


21

Benzothiazines - The acidic 4-hydroxy-2-methyl-2H-1,2-benzothiazine-3-carboxanilide 1,1-dioxide (22, R=H) had about twice the potency (CE) of phenylbutazone.[70] The major urinary metabolite in man (22, R=OH) had nearly twice the plasma half-life of the parent compound,[71] but was reported to have weak anti-edema activity in the rat.[72] Compound 23, a member of a potent series of isomeric 1,2-benzothiazine 1,1-dioxide AI agents had 1.5 times the potency (CE) of indomethacin.[73]



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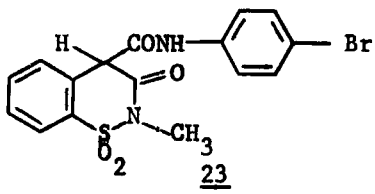
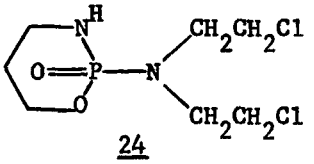


23

Gold - The parenteral use of gold salts in RA has received prominent attention in the past year, and it has been concluded that chrysotherapy is beneficial in active rheumatoid disease.[74] New impetus for research in this area may have been provided by the discovery of an orally effective agent in the adjuvant arthritis assay. Triethylphosphinogold chloride (p.o.) was as effective as gold sodium thiomalate (i.m.) at equal gold doses, and produced no overt kidney damage following chronic administration.[75] Gold salts may exert their beneficial effects by preventing protein denaturation[76] and/or modifying the immune response.[9,77]

Prostaglandins - In spite of the accumulating evidence for the pro-inflammatory nature of prostaglandins (see Modes of Action), PGE_1 and PGE_2 have been shown to have antiarthritic properties when given s.c. to rats at daily doses of about 1 mg/rat.[78,79] Glenn and Rohloff have suggested[79] that the antiarthritic effects of the prostaglandins may be related to the concomitant adrenal hyperplasia, prostration, and diarrhea caused by the high pharmacologic doses used.

Immunosuppressives - It has been generally accepted that RA is an example of an immune complex disease,[80,81] and so clinical trials of cytotoxic immunosuppressive agents such as chlorambucil,[82,83] azathioprine,[84,85] cyclophosphamide (24),[86] and isophosphamide (25),[87] have continued. The risk of severe side effects, however, has resulted in a recommendation[88] that these drugs be used only in closely supervised treatment of patients who are unresponsive to conventional therapy. The possibility that the cytotoxic agents may act by predominantly antiinflammatory rather than immunosuppressive mechanisms has been discussed.[89]



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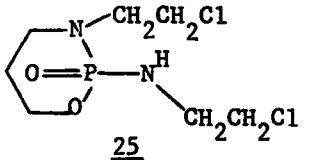
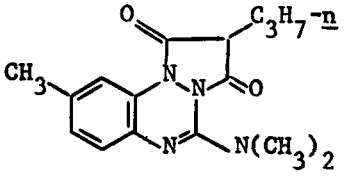


25

Miscellaneous - Clinical experiences with apazone (26) have been summarized.[90] Good AI activity, with little incidence of side effects, was noted in patients with a variety of inflammatory diseases receiving 26 at 0.4-1.6 g/day. Tinoridine HCl (27) was introduced on the market in Japan as an antiinflammatory-analgetic agent.[91] The benzophenone (28) had about twice the potency (CE) of phenylbutazone.[92]



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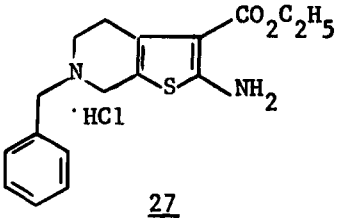

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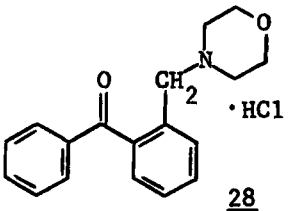
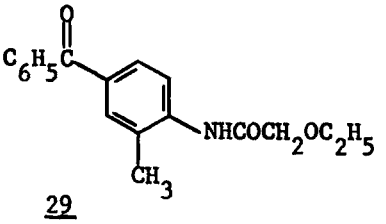
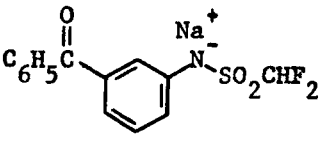


28

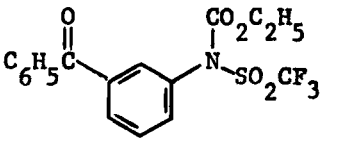
Compound 29 proved comparable to phenylbutazone (CE).[93] Additional reports have appeared on the AI activities of diflumidone sodium (30)[94] and triflumidate (31)[95] in acute and chronic rodent screens.



29

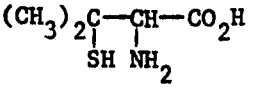


30

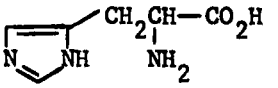


31

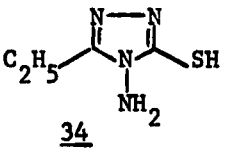
Benefit has been claimed for patients with RA who were treated with D-penicillamine (32)[96] and L-histidine (33).[97] The triazole 34 had comparable AI activity (CE) to phenylbutazone.[98]



32



33



34

Comment - Our overall impression is that there have been no really significant advances in the past year. An encouraging sign, however, is the increasing amount of research which is being directed toward a better understanding of the inflammatory processes. We hope that from this work will come the breakthrough so badly needed in this field.

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Section V - Topics in Biology

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Chapter 20. Mechanisms of Resistance to Antibiotics

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Introduction - The search for new antimicrobial agents, either from natural sources or by the chemical or biochemical modification of existing compounds is predicated by the desire to obtain agents with a) broad spectra of bacterial effectiveness, b) low toxicity, c) activity against naturally occurring resistant organisms. Thousands of antibiotic derivatives have been prepared using numerous approaches to structure-activity relationships, which are still largely empirical. Comparatively few antibiotics have been modified successfully as the result of rational biochemical predictions. It is now clear that natural resistance mechanisms, for all their clinical problems, do provide some clues about structure-activity relationships and one can derive useful conclusions about functional groups important to biological activity.

The occurrence of resistance strains - It is easy to generate bacterial mutants which are resistant to antibiotics; biochemical studies of such mutants have provided much information on cell structure and metabolism[1]. These mutants arise by single or multiple-changes in chromosomes and the properties are inherited in Mendelian fashion. However, the resistant strains which are isolated from natural sources (during antibiotic therapy in humans and animals) rarely correspond to the types mentioned above[2,3]. Clinical isolates of both gram-positive and gram-negative bacteria are often resistant to unrelated groups of antibiotics, are not produced by mutation of the parent sensitive strains, and the resistance characters are inherited in non-Mendelian fashion[4]. The resistant strains carry blocks of genes which determine resistance to a variety of antibiotics; these blocks of genes can be transferred from strain to strain by the processes of conjugation or transduction. The complete extrachromosomal unit is usually referred to as a resistance factor (R-factor) in gram-negative organisms and as a plasmid in gram-positive organisms. It is most likely that these resistance mechanisms existed within the bacterial population before the advent of antibiotics, and were selected when antibiotics were used in quantity[5]. Since "naturally-occurring" resistance is of greatest significance in terms of the medical uses of antimicrobial agents this review will concentrate on this form of resistance.

General mechanisms of resistance - In 1952, Davis and Maas discussed the ways by which an organism might become resistant to an antibiotic[6]. These mechanisms are outlined in Table 1, together with examples of each type.

Table 1
Biochemical mechanisms of drug resistance

Mechanism	Examples	
	Clinical* Isolates	Laboratory** Isolates
Inactivation of drug by bond cleavage or modification	R-factor or plasmid determined inactivation of β-lactam or aminoglycoside antibiotics	?
Modification of drug-sensitive site	erythromycin resistance in S. aureus (altered ribosomes)	streptomycin, spectinomycin resistance (altered ribosomes)
	sulfonamide resistance in Pneumococci (altered enzyme)	rifamycin (altered RNA polymerase) sulfonamide resistance in Pneumococci.
Loss of permeability to drug	R-factor determined resistance to tetracycline or sulfonamides	resistant strains produced by serial transfer?
Increased levels of enzyme inhibited by drug		5-methyltryptophan resistance, Psicofuranine resistance
New biochemical pathway which bypasses inhibited reaction		
Increased concentration of a metabolite that antagonizes the inhibitor		sulfonamide resistance?
Decreased requirement for product of inhibited reaction		sulfonamide resistance?

* refers to mechanisms of resistance in bacteria which appear during therapeutic use of an antibiotic.

** refers to mechanisms of resistance in bacterial strains isolated under laboratory conditions. e.g. deliberate selection of antibiotic resistance.

The mechanisms favoured by "naturally-resistant" organisms involve drug inactivation (either by bond cleavage or derivatization), an altered drug receptor site, or reduced permeability to the drug.

Studies of laboratory-derived (single-step) resistant mutants have proved to be of considerable biochemical utility. For example, streptomycin-resistant mutants have an altered receptor site due to a change in a protein of the small ribosomal subunit, while erythromycin-resistant mutants have a change in a protein of the large ribosomal subunit[7]. Altered RNA polymerases are found in mutants resistant to rifampicin and streptolydigin[8,9]. Fusidic acid resistance occurs as a result of mutations which alter a factor required in protein synthesis[10]. Such forms of antibiotic resistance have been useful in determining the mode of action of the antibiotic and in studies of macromolecular structure and function. By contrast, in "natural" resistance, the resistance mechanism is, in most cases, unrelated to the biochemical mode of action of the drug. The antibiotics with known plasmid-determined resistance are listed in Table 2.

Table 2

R-Factor or plasmid-determined resistance to antimicrobial agents *

tetracycline	neomycin
chloramphenicol	kanamycin
sulfonamides	streptomycin
erythromycin	spectinomycin
lincomycin	gentamicin
penicillins	tobramycin
cephalosporins	?fusidic acid

* Plasmid-determined resistance has not been found to nalidixic acid, nitrofurans, polymixin, bacitracin, or rifampicin.

Specific mechanisms of resistance

A. Sulfonamide resistance is widespread among clinical isolates of bacterial strains, and it was the appearance of sulfonamide resistance in Japan in the 1950's, which provided the first example of an "epidemic" of resistance[11]. Within 5 years after the introduction of sulfonamides for the treatment of bacillary dysentery, 80-90% of the Shigella dysenteriae isolates were found to be resistant to these drugs. A large proportion of these strains was found to be resistant to other unrelated antibiotics such as streptomycin and chloramphenicol, and genetic studies led to the discovery of transmissible drug resistance characterized by the presence of R-factors or plasmids. R-factor or plasmid-determined sulfonamide resistance is now common among bacterial pathogens. The biochemical mechanism of sulfonamide resistance in these strains has been little studied; it is currently accepted that the R^+ strains (strain carrying the R-factor) have a decreased permeability for the drug, although the biochemical evidence for such a mechanism is far from convincing[11].

Not all sulfonamide resistance is determined by R-factors or

plasmids. Genetic alterations in the dihydropteroic acid-forming enzymes have been found in some resistant strains of Pneumococci[12]; the sulfonamide drugs do not act as competitive inhibitors of tetrahydrofolate formation in such strains.

B. Tetracycline resistance is probably the most common form of R-factor-determined resistance. As far as can be ascertained all tetracycline-resistant strains obtained from clinical situations are resistant because they carry an R-factor or a plasmid. It has been concluded that tetracycline resistant strains are impermeable to the drug, although the evidence is not convincing[13]. Biochemical studies indicate that tetracycline is actively transported into sensitive cells which implies that resistance to the drug is due to inactivation of a specific transport system for tetracycline. (It seems strange that bacteria would want to take up such a drug!) R^+ strains possess a basal level of resistance to tetracycline which is enhanced when the cells are grown in the presence of the drug; some evidence for an inducible genetic system has been provided but detailed genetic and biochemical analysis will be necessary to establish this mechanism[14].

The tetracyclines have been used extensively for agricultural purposes as animal feed additives; however, their use has been discontinued in England on evidence of the report of the Swann committee[15]. This recommendation was based on findings that the bacterial flora of animals fed tetracyclines (and other antibiotics) on a continuous basis contained a high proportion of strains carrying R-factors. Such animals constitute a reservoir of resistant strains which may be passed to human subjects and possibly transmit their resistance characters to antibiotic sensitive pathogens. There is no direct evidence that such transfer occurs, but there is little doubt that the extensive use of antibiotics selects for an antibiotic-resistant population of bacteria among cattle, poultry, swine and fish which have been maintained on antibiotic feed additives[16].

There is strong genetic and biochemical evidence that the gene for tetracycline resistance is closely linked to the resistance transfer factor (RTF, the portion of the R-factor which determines transmissibility). Indeed, when the R-factor dissociates, the gene for tetracycline resistance associates with the RTF and not the r-determinants[17]. This would imply that selection for tetracycline resistance must automatically select for the ability to transfer. If transferability is considered to play an important role in the development of antibiotic resistance in human pathogens, the use of tetracycline must certainly be restricted. However, the present state of our knowledge cannot allow us to say whether transmissibility is a factor in the increase in occurrence of antibiotic-resistant pathogens. Cross-resistance to all the tetracycline antibiotics is common; one possible exception is minocycline, a semisynthetic tetracycline which has some activity against tetracycline resistant strains[18].

C. Resistance to β-lactam antibiotics (penicillins and cephalosporins) has been extensively studied, since it has significantly dictated the application and use of antibiotics of this class. With one notable exception (methicillin resistance in Staphylococcus) resistance to these antibiotics

is due to the production of a β-lactamse (penicillinase) which inactivates the antibiotics by opening the β-lactam ring[19]. There appear to be a number of β-lactamases which show a broad spectrum of activity on the β-lactam antibiotics in clinical use; none of these antibiotics are completely inert[20]. Resistance to the β-lactams in Staphylococcus is determined by a plasmid; substantial genetic and biochemical data have been accumulated in this system, notably by Novick[21], and by Richmond[22], and their collaborators. β-Lactamase production in Staphylococcus is inducible (requires the presence of antibiotic for expression) and very low (subinhibitory) concentrations of a β-lactam antibiotic are sufficient to induce maximal expression of the gene(s) for the β-lactamase.

The problem of resistance to the β-lactam antibiotics was originally restricted to gram-positive strains. The discovery and use of β-lactam antibiotics with activity against gram-negative strains (ampicillin, carbenicillin, cephalosporins) has led to the appearance of strains of E. coli, P. aeruginosa and K. pneumoniae, etc. which are resistant to the β-lactam antibiotics.

In almost all gram-negative strains, resistance is due to the R-factor determined production of a β-lactamase[23]. Unlike the β-lactamase in gram-positive strains the enzyme is produced constitutively (does not require the presence of antibiotic to induce formation of the enzyme). In Staphylococcus the β-lactamase is released into the medium, but the β-lactamase of enteric bacteria appears to belong to the class of periplasmic enzymes, which are situated between the cell wall and the cell membrane[24].

The existence of carbenicillin-resistant strains of Pseudomonas aeruginosa, although not widespread, is of considerable concern in burn clinics since carbenicillin has proved to be an effective agent in the treatment of Pseudomonas infections. Studies have shown that drug resistance is transferable (R-factor-determined) in many of these instances and there is circumstantial evidence that the Pseudomonas strains acquired this resistance by accepting R-factors from strains of Proteus or Klebsiella which were present in the flora of burns[25]. Lowbury and coworkers have demonstrated R-factor transfer in infected burns in mice; this is the first evidence which emphasizes the importance of both transfer and selection in the development of resistance[26]. Not all strains of Pseudomonas carry R-factors, and an inducible β-lactamase (similar to that in gram-positives?) has been reported in some isolates. The β-lactamase which is R-factor determined and which is responsible for carbenicillin resistance in burn clinics is, like other R-factor-determined enzymes, produced constitutively[27].

Large numbers of penicillin and cephalosporin antibiotics have been made in efforts to expand the spectrum of these drugs and to produce antibiotics resistant to the action of β-lactamases. It seems unlikely that a β-lactam antibiotic which is completely refractory to the action of β-lactamases will be found. This pessimism is based on the following argument: the β-lactam ring is critical to antibiotic activity and cannot

be modified. Substances introduced adjacent to the β-lactam ring can change the effectiveness of the molecule as a substrate for β-lactamase, but cannot completely eliminate this activity. The range of known β-lactamases makes it probable that any modified β-lactam is going to be a substrate for one of the enzymes present in nature and the subsequent use of this antibiotic will select for resistant strains producing the most effective enzyme. Methicillin-resistance in Staphylococcus aureus is not associated with β-lactamase production. It seems most likely, in view of the rather unusual properties of these strains (co-resistance to cephalosporin), that resistance is due to impermeability to the antibiotic[28].

D. Chloramphenicol resistance has been extensively studied by Shaw[29], and by Okamoto and Suzuki[30], and is due to inactivation of the drug by O-acetylation[31] in enteric bacteria and Staphylococcus. The enzyme, chloramphenicol acetyltransferase is determined by an R-factor in the Enterobacteriacae and by a plasmid in Staphylococcus; it is produced constitutively in E. coli and its production is induced by chloramphenicol in Staphylococcus aureus[31]. Chloramphenicol usage is tightly controlled and it is surprising, perhaps, that a large proportion of multiply resistant organisms isolated from nature are chloramphenicol resistant. Until recently, no other type of chloramphenicol resistance had been recognized although Nagai and Mitsuhashi have reported the isolation of a number of chloramphenicol-resistant R^+ strains which seem to be impermeable to the antibiotic[32].

E. Aminoglycoside antibiotics have several well-characterized modes of enzymatic inactivation and all clinical isolates of bacteria resistant to these antibiotics have been shown to possess these inactivation mechanisms[33]. Some seven different enzymes have been characterized which inactivate one or more of the aminoglycoside antibiotics (Table 3). Streptomycin (and spectinomycin) adenylylation was apparently the first mechanism to be recognized (selected) and most multiply resistant strains, isolated after 1956, carry this form of resistance. This resistance determinant has been detected in strains lyophilized in 1945, which suggests that R-factors were present before the extensive clinical use of antibiotics[5]. Shortly after the introduction of kanamycin as a broad-spectrum antibiotic, a number of strains carrying neomycin and kanamycin resistance were isolated[34]. Such resistance is now quite widespread and inactivation of neomycin and kanamycin by phosphorylation is a common form of aminoglycoside resistance in E. coli, P. aeruginosa and K. pneumoniae. Inactivation of kanamycin by acetylation of the 6-amino group of the glucosamine ring has been demonstrated in some strains. This resistance mechanism gives a low level of resistance and is rare among clinical isolates.

Gentamicin, the newest of the aminoglycoside antibiotics to be used therapeutically, has proved to be a successful drug[35]; for one reason, it is effective against neomycin and kanamycin-resistant strains, such as many Pseudomonads. However, two forms of gentamicin inactivation have been found in gentamicin-resistant strains isolated in hospitals and clinics using the drug.

Table 3

Antibiotic	Inactivation Mechanism	Substrates	Resistance Spectrum
streptomycin phosphotransferase	phosphorylation of L-glucosamine ring	streptomycin,	streptomycin
streptomycin	adenylylation of L-glucosamine ring	streptomycin, spectinomycin,	streptomycin spectinomycin
kanamycin acetyltransferase	acetylation of D-glucosamine ring	kanamycin A, B, neomycin, gentamicin	kanamycin A, (low level resistance to other substrates)
neomycin-kanamycin phosphotransferase I	phosphorylation of D-glucosamine ring	neomycin, kanamycin, paromomycin	neomycin, kanamycin, paromomycin
neomycin-kanamycin phosphotransferase II	phosphorylation of D-glucosamine ring	neomycin, kanamycin, paromomycin, ambutyrosine	neomycin, kanamycin, paromomycin, ambutyrosine
gentamicin adenylate synthetase	adenylylation of garosamine ring	gentamicin, kanamycin	gentamicin, kanamycin, tobramycin
gentamicin acetyl transferase	acetylation of deoxystreptamine ring	gentamicin C	gentamicin C

Gentamicin resistant strains of E. coli and Klebsiella pneumoniae have been reported in hospitals in the U.S.A.[36,37] and France[38]. The antibiotic is inactivated by adenylylation; kanamycin and tobramycin can be inactivated by the same mechanism[39]. The enzyme, gentamicin adenylylate synthetase, is determined by an R-factor gene. Structural studies with dideoxykanamycin which had been inactivated by this enzyme have shown that the adenyl residue is on the 2"OH of the kanosamine ring[40]. In vivo transfer of the R-factor to species other than E. coli and Klebsiella has not been reported. This adenylylating enzyme has provided the basis for a rapid and sensitive assay for gentamicin in biological fluids[41,42].

Gentamicin resistance in Pseudomonas aeruginosa occurs as a result of acetylation of the 3-amino group of the deoxystreptamine ring, a reaction which seems to be specific to the gentamicin C antibiotics [43,44]. This is the first report of modification of the deoxystreptamine moiety in any antibiotic, which is somewhat surprising since this sugar is common to the aminoglycoside antibiotics. This resistance mechanism has not been found in strains other than P. aeruginosa and it has not been demonstrated to be transmissible. By contrast, carbenicillin resistance, and neomycin-kanamycin resistance in P. aeruginosa can be conjugally transferred to E. coli.

An attractive feature of gentamicin and tobramycin[45] (3-deoxykanamycin B) is that they are structurally incapable of inactivation by the ubiquitous neomycin-kanamycin phosphorylating enzymes, which modify the 3'-OH group of the D-glucosamine ring. The presence of these enzymes is probably the reason why most clinical isolates of Pseudomonas aeruginosa are resistant to neomycin-kanamycin. Gentamicin and tobramycin contain a 3'-deoxy sugar that lacks the site modified by the phosphorylating enzymes; as an additional bonus they can inhibit the phosphorylation of other antibiotics[46]. The occurrence of such aminoglycoside antibiotics has indicated the possibility of structural modifications of aminoglycosides with the group modified by the inactivation removed without reducing the potency of the antibiotic. The aminoglycosides offer great potential in this respect and may have an advantage over the β-lactam antibiotics, where the site of enzymatic inactivation cannot be removed. The production of synthetic and semisynthetic aminoglycoside antibiotics may allow one to keep ahead of nature's ability to produce inactivating enzymes. Marked success has been reported by Umezawa and his co-workers[47] who have synthesized 3,4-dideoxykanamycin B, a close derivative of gentamicin and tobramycin, which is effective against a number of kanamycin resistant organisms including Pseudomonas. It is to be hoped that similar developments in this area will be forthcoming. Would an aminoglycoside still be an antibiotic, when all of its enzymatically modifiable functions are removed?

F. Macrolide and lincosamide resistance in Staphylococcus is the only naturally-occurring resistance mechanism which appears to involve an alteration in the target site of the antibiotic, rather than a change in the antibiotic itself. Considering erythromycin and lincomycin as typical antibiotics, two types of resistance can be considered: a) inducible resistance, in which the presence of sub-inhibitory concentrations of erythromycin can induce resistance to both erythromycin and lincomycin and b) constitutive resistance, in which strains are resistant to both classes of drug without requiring previous exposure to erythromycin or lincomycin. Lincomycin resistance without concomitant erythromycin resistance is rare in clinical isolates. The work of Weisblum[48], and Mitsuhashi[49], and their colleagues has shown that inducible and constitutive erythromycin resistance result from a change in the ribosome, such that the drugs are incapable of binding at their target site. Recent work by Lai and Weisblum[50] has shown that erythromycin resistant strains of S. aureus contain an enzyme which methylates specific sites on the RNA of the 50S ribosomal subunit. Ribosomes containing the unmethylated RNA can bind the antibiotic, but ribosomes with methylated RNA are refractory to the antibiotic. Erythromycin resistance in Streptococcus pyogenes may also be the result of methylation of ribosomal RNA[51]. The evidence suggests that erythromycin-lincomycin resistance in Staphylococcus is determined by plasmid genes[52].

Conclusion - Mechanisms of resistance to the common, clinically-used antibiotics are summarized in Table 4.

Table 4

Mechanisms of resistance in bacteria

Antimicrobial Agent		
Penicillins, Cephalosporins	β-lactamase	ring cleavage
Streptomycin	kinase	phosphorylation
Streptomycin, Spectinomycin	adenylyltransferase	adenylylation
Kanamycin Δ	acetyltransferase	acetylation
Kanamycin, Neomycin, Paramomycin	kinase	phosphorylation
Gentamicin	acetyltransferase	acetylation
Gentamicin, Kanamycin	adenylyltransferase	adenylylation
Chloramphenicol	acetyltransferase	acetylation
Erythromycin, Lincomycin	methylase	methylation of ribosomal RNA

Δ Acetylation of neomycin and gentamicin also occur, but these antibiotics are not completely inactivated by this form of modification.

The infectious diseases clinician wants antibiotics of broad spectrum and low toxicity. Unfortunately, little is known or can be done about the latter. With respect to spectrum, it is in the hands of the pharmaceutical houses and physicians to decide whether broad spectrum antibiotics remain as such, or if they develop reduced spectra as a result of indiscriminate use with consequent selection of resistant organisms. R-factors are probably the most common form of resistance determinant in natural isolates, and the promiscuity of this agent is undoubtedly related to its widespread occurrence in clinically-isolated strains[53]. A large number of strains are known to be capable of carrying R-factors (Table 5).

Table 5

Bacterial species known to carry R-factors

Escherichia coli, *Escherichia freundii*
Salmonella typhimurium, *Salmonella typhosa*
Shigella dysenteriae, *Shigella flexneri*
Pseudomonas aeruginosa
Proteus mirabilus
Serratia marcescens
Aerobacter aerogenes
Klebsiella pneumoniae
Vibrio cholerae
Pasteurella sp.
Citrobacter

There seems to be little hope that agents will be developed to prevent the strain-to-strain transfer, or promote the segregation (curing) of R-factors. Therefore, the proportion of resistant bacterial strains in nature must be kept to a minimum by the carefully controlled use of antibiotics, which might include rotating use of antibiotics with quite different resistance mechanisms. There is good evidence that R^+ resistant strains are outgrown by the sensitive, wild-type bacterial flora when the selecting antibiotic is withdrawn from use[53].

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Chapter 21 - The Serum Complement System

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Introduction - Complement was discovered in the late nineteenth century when it was recognized that the bactericidal activity of fresh serum required the participation of at least two factors: a heat-stable factor, specific for the particular organism (antibody); and a second, non-specific, heat-labile factor designated complement. During the two decades following the discovery of complement, it became apparent that complement did, in fact, consist of several components which act in a definite sequence to effect lysis and death of the bacteria. In the 1950's, the development of sophisticated methods in protein chemistry and suitable functional assays for individual complement components facilitated the elucidation of the chemical, physical, and functional properties of the complement system. As a result, the serum complement system is now known to consist of nine distinct proteins that interact to mediate and amplify many of the biological effects of immune reactions (1,2).

It should be noted that a discussion of the complement system as an entity separate from the balance of the plasma proteins is somewhat artificial, albeit convenient. Plasma contains several multicomponent enzymatic systems which interdigitate to form a complex functional unit. This functional unit, in turn, interacts with cellular elements and/or with exogenous agents to effect a multiplicity of homeostatic, protective, and occasionally injurious events. For instance, recent studies have demonstrated the intimate connections between components of the coagulation mechanism, the kinin-generating system, and complement (3,4). Furthermore, it has been recognized that other non-immunologic systems, such as bacterial proteases and lysosomal enzymes, can initiate complement activation (5).

The purpose of this report is to review briefly some of the pathophysiological and homeostatic functions of the complement system and to indicate some of the clinical conditions in which pharmacologic regulation of complement activity might offer a rational approach to the therapy of allergic and immunologic disease.

Nomenclature - For a number of years, a great deal of confusion had been generated by the lack of agreement on a consistent language for describing the complement system. In 1968, a standardized nomenclature was agreed upon (6). According to this nomenclature, the individual complement components are designated numerically in the order of their reaction sequence as follows: C1, C4, C2, C3, C5, C6, C7, C8, C9. Since initially an understanding of the functional properties of the complement components derived from studies of immune hemolysis, a convention was established for designating the reaction sequence and intermediate complexes on cell surfaces. E represents erythrocytes; A, antibody directed against the erythrocytes; and the intermediates indicated by EAC, followed by the numbers corresponding to the individual components that have reacted with EA. For

example, EAC142 describes an intermediate consisting of erythrocytes sensitized with antibody and containing the first, fourth, and second components of complement. Activated forms of the components are designated by a bar above the symbol; e.g., activation of C1 to $\overline{C1}$. Fragments of the components are indicated by a lower case letter; e.g., C3a, C3b, C3c, and C3d.

Physicochemical Properties of Complement - The first component of complement (C1) is a macromolecule of molecular weight approximately 1×10^6 which can be dissociated in the presence of sodium EDTA into three distinct protein subunits designated C1q, C1r, and C1s (7). C1q, the largest of the three with a molecular weight of about 400,000, electrophoretic mobility in the gamma region, and a sedimentation constant of 11S, has been visualized in the electronmicroscope as a coiled chain of six to eight globular subunits (8). C1q is the portion of the C1 macromolecule that possesses the binding site capable of interacting immunoglobulin molecules; namely, IgG (principally of the $gamma_1$, $gamma_2$, and $gamma_3$ subclasses) and most IgM molecules; but not with IgA, IgD, or IgE. The amino acid composition and carbohydrate content of C1q are similar to those of basement membrane proteins (9). C1q can be quantitated in serum by functional and immunochemical methods. The concentration in normal human serum is approximately 20 mg%. C1r, a 7S protein with beta mobility, normally exists in serum non-covalently bound to C1q. C1q probably activates C1r by a mechanism as yet poorly understood (10), and the C1r, in turn, activates C1s (11), altering its electrophoretic mobility and converting the proesterase to an active esterolytic enzyme (12). The esterolytic activity of $\overline{C1s}$ can be inhibited by phosphous esters (13), as well as by the serum protein inhibitor of C1. C1s, an alpha-2 protein, molecular weight 80,000, present in normal serum at a concentration of approximately 12 mg%, possess the active enzymatic site(s) of the $\overline{C1}$ molecule which facilitate the cleavage of the natural substrates of this enzyme, C4 and C2 (14).

The binding and cleavage of C4, a beta globulin of molecular weight approximately 250,000, by $\overline{C1}$ apparently uncovers another enzymatic site on the C1s fragment which effects the cleavage of the C2 molecule into at least two fragments (15). One of the C2 fragments (C2a) can combine with a fragment of C4 (C4b) to form a new enzyme, C3 convertase ($\overline{C42}$) (15). This $\overline{C42}$ enzyme is extremely unstable under physiologic conditions; but if C3 is available, it splits the C3 into two fragments, C3a and C3b. C3, a genetically polymorphic protein, is the complement component present in highest concentration in normal serum (150 ± 50 mg%). Immunochemical quantitation of this protein is relatively simple. Hence, most clinical laboratories generally assess abnormalities of complement in disease states by measurements of C3. As a consequence of cleavage of C3 by the $\overline{C42}$ enzyme, a fragment of the molecule (C3b) acquires proteolytic enzymatic activity that probably is involved in cleavage of the next component in the series, C5 (5). C5, another beta globulin, has a molecular weight of about 180,000, and a sedimentation rate of 9S (16). This protein normally is present in serum at a concentration of about 8 mg%. C5 combines with C6 and C7 to form an active complex capable of interacting with C8 and C9 (17).

The characteristics of several other proteins intimately associated with the complement system should also be mentioned. These include the naturally occurring inhibitors of the complement system, the C1 inhibitor and the C3 inhibitor. The C1 inhibitor is an alpha-2 protein normally present in serum at a concentration of 18 ± 4 mg%. The protein has a molecular weight of approximately 100,000 and a sedimentation rate of 3S. It is capable of inhibiting the esterolytic and hemolytic activities of C1 stoichiometrically, but apparently does not interact with the precursor form of the C1 molecule. Interestingly, the C1 inhibitor is also capable of blocking the activity of several other proteins; namely, plasmin, kallikrein, and a permeability factor designated PF/dil (18). The C3 inhibitor is a 5S heat-stable beta-globulin that is capable of blocking the activity of C3b (19). Some of the physicochemical properties and the concentrations of each of the complement proteins in normal human serum are given in Table I:

Properties of the Complement Components

Complement Component	Estimated MW($\times 10^{-3}$)	Sedimentation Coefficient(S)	Relative Electrophoretic Mobility	Normal Serum Conc. Hemol. Eff. Mol.($\times 10^{-12}$)**	mg%
C1	1000	19.0	-	40	--
C1q	400	11.0	$\gamma 2$	--	20
C1r	150	7.0	β	--	--
C1s	80	4.0	$\alpha 2$	--	12
C2	117	5.5	$\beta 2$	5	3
C3	185	9.5	$\beta 1$	2	150
C4	240	10.0	$\beta 1$	40	40
C5	180	9.0	$\beta 1$	2	7.5
C6	95	5.6	$\beta 2$	2	--
C7	110	5.6	$\beta 2$	--	--
C8	150	8.0	$\gamma 1$	--	1
C9	79	4.5	α	5	1

Reaction Sequence - There are several pathways by which all or part of the complement system can be activated. The nexus of these various activation pathways, the C3 molecule, and the later-acting complement components, yield the bulk of the biologically active components of the complement system. However, as will be described shortly, some of the biological effects of complement are the consequence of the action of C1, C4, and C2 alone.

The classical complement activation sequence is initiated by the binding of C1 to an antigen-antibody complex. A single IgM in complex

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*Serum proteins have been separated according to their electrophoretic mobilities in a standard buffer at pH 8.6. In this procedure, albumin is the most rapidly migrating, and gamma globulin the slowest serum protein.

**Hemolytically effective molecules assayed in whole serum using standard functional assays for the individual complement components (2). Normal values for some of the components have not yet been established.

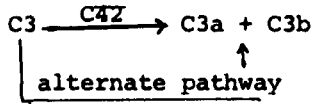
with its corresponding antigen is capable of binding one C1 molecule; whereas at least two IgG molecules of the appropriate subclass are required for C1 fixation (20). As a consequence of binding, the C1 undergoes a conformational change and an autocatalytic activation. The activated $\overline{C1}$ is then capable of cleaving C4 and C2, forming the $\overline{C42}$ complex. Interaction of the active $\overline{C423}$ with native C5 generates an unstable intermediate $\overline{C4235}$ which then acts on C6 and C7. Subsequently, C8 and C9 are bound and activated.

It should be noted that this pathway can be activated by non-immunologic mechanisms as well. For example, it has been demonstrated that a protein active in the clotting mechanism, plasmin (the activated form of plasminogen), is capable of activating C1 to $\overline{C1}$, thus initiating the classical complement sequence(3). Likewise, precipitation of C1 with highly charged molecules, such as carrageenin, also results in C1 activation (21). In addition, trypsin, lysosomal enzymes, and bacterial proteases can directly activate the complement system without the participation of specific immune complexes (5).

A number of years ago, a major alternate pathway of complement activation was described. This pathway, initiated by incubation of endotoxin or yeast with fresh serum, is mediated by the properdin system, bypassing the early complement components (C1, C4, and C2) to effect the activation of C3 directly (22). Properdin and at least two proteins designated Factors A and B constitute the principal components of this bypass-activating system. Two of these proteins have recently been isolated: properdin, a euglobulin with a molecular weight of 280,000 (23); and Factor B (previously designated GBG), a genetically polymorphic pseudoglobulin (24). Recently, it has been shown that certain immunoglobulins (guinea pig gamma$_1$, human IgA, and possibly IgE) that do not fix C1 are capable of initiating the "bypass" sequence of complement activation (25,26). It is not clear at the present time whether these antibodies activate the classical properdin system or activate still another alternate pathway.

Biological Effects of Complement - Activation of the complement system under appropriate conditions leads to the generation of a number of biologically active fragments that participate in the inflammatory response. Activation of all nine of the components results in membrane damage (i.e., cytolysis, bacteriolysis, and possibly the immunopathogenic lesions of immune complex disease).

Anaphylatoxins and Chemotactic Factors (cf, reference 5 for extensive review of this area) - There are two complement-associated anaphylatoxic factors which effect smooth muscle contraction and increase vascular permeability. The first of these, a polypeptide of molecular weight approximately 7,000, C3a, arises from the cleavage of C3 in the following reactions:



The second anaphylatoxic factor is generated from C5 by the following reaction:

$$C5 \xrightarrow{\overline{C423}} C5a + C5b$$

C5a is a fragment of the parent molecule with a molecular weight of about 10,000. The actions of both anaphylatoxins can be blocked by antihistamines, but apparently the biological effects of each operate via separate mechanisms since smooth muscle no longer sensitive to C3a can be stimulated by C5a. It should be noted that response of smooth muscle to anaphylatoxin exhibits tachyphylaxis; whereas the response to histamine does not. It is clear, however, that activation of the complement system at least through C3 leads to complement-dependent histamine release. This phenomenon is mediated by immunoglobulins other than IgE.

Both C3a and C5a have chemotactic activities as well (27,28). That is, they are capable of promoting migration of white blood cells to a site of inflammation. It is not clear whether the C5a and/or C3a fragments responsible for chemotaxis are chemically identical with the corresponding anaphylatoxin molecules. The precise mechanism by which chemotaxis is accomplished is only poorly understood at the present time, but it does appear that at least two serine esterases supplied by the polymorphonuclear leukocytes are required for this response to chemotactic factors.

Immune Adherence and Opsonization - It is known that, although phagocytosis can proceed in the absence of complement, this process is greatly accelerated by an intact complement system (29). Phagocytosis of IgM antibody-antigen complexes requires complement. IgG, on the other hand, by itself promotes phagocytosis, but this effect is augmented by the fixation and activation of complement. Based on experimental evidence, as well as clinical observations, it is now clear that immune adherence and opsonization require activation and uptake of complement (at least through C3b). It has been suggested that receptors on phagocytes capable of recognizing C3 in effect cement a particle to the white cell, thereby facilitating both phenomena. There is evidence, however, that the fixation of C5 may also be required for phagocytosis of certain microorganisms, such as monilia (30).

Membrane Damage - Activation of the complement system at discrete sites on cell surfaces results in irreversible damage to a cell membrane, leading to lysis of the cell. Red blood cells, most if not all nucleated cells, and many bacteria are susceptible to the lytic action of complement. The action of complement on biological membranes results in characteristic electronmicroscopically identifiable lesions. These lesions, demonstrated with negative-staining techniques, are approximately 80 Å in diameter (31). Recent evidence suggests that the lesions appear after the complement reaction sequence has proceeded through C5 (32), although there are claims that all nine components are required. In any case, a functional lesion in the membrane is not detectable until at least the first eight components have been activated. This functional lesion leads to a disruption of the

osmotic integrity of the cell and a sequential loss of small ions, amino acids, polypeptides, and macromolecules (33). In nucleated cells, there is, in addition, a profound enzymatic cleavage of DNA following immune cytolysis (34).

Coagulation - Recent evidence derived from studies of rabbits deficient in C6 indicated that the clotting mechanism is facilitated by this component, since C6-deficient rabbit plasma showed a defect in coagulation that could be restored to normal by the addition of C6 (35).

Virus Neutralization - The neutralization of viruses under some conditions requires the participation of anti-viral antibody and at least some of the complement components. For example, it has been shown that neutralization of herpes virus by IgM anti-herpes antibody requires the activation and binding of C1 and C4 to the virus particle. Smaller amounts of C4 will suffice if C3 is available (36).

Ontogeny, Phylogeny, and Biosynthesis of Complement - Fluid phase, heat-labile hemolytic factors have been identified in primative invertebrates (37). However, a clearly defined complement system appears phylogenetically first in cartilagenous fish. So far, of the vertebrates that have been studied, all have complement, although the amount as detected in a standard hemolytic assay varies widely from one species to another. Many of the components are immunochemically and physicochemically species specific. Nonetheless, several components are sufficiently similar functionally to permit interaction of a component of one species with that of another. In short, phylogenetic studies, while only fragmentary at the present time, do indicate that the complement system arose early in evolution and that the basic structural and functional properties of many of the components have undergone relatively little genetic change. A significant exception to this general rule can be found in the genetic polymorphism of a few of the components, the principal example being C3 (38). By means of electrophoretic analysis, C3 has been shown to exist in two common genetically determined forms, designated S for the slower or cathodal type and F for the faster or anodal type. A number of rare variants of S and F alleles have been described. These variant molecules, while functionally indistinguishable, must have resulted from minor amino acid substitutions and, therefore, are reflections of some degree of evolutionary variability. A similar polymorphism of C4 has been described, but the exact genetic basis for this has not been completely clarified (39). The discovery of polymorphic form of the complement proteins has been of considerable importance in one approach to the study of the ontogeny and biosynthesis of complement.

According to a number of independent lines of investigation, complement biosynthesis by the fetus is initiated early in gestation (40,41). It has been shown that C3 and C4, as well as whole complement activity, are present in fetal serum and, furthermore, that the electrophoretic variants of C3 in fetal sera may differ from the maternal type (42), suggesting a lack of transplacental passage and thus active fetal synthesis of this component. In addition, human fetal tissues are capable of synthesizing in vitro biologically active C1 through C5 (the components that have been

been studied thus far). Recently, the sites of synthesis of most of the individual complement components have been identified in adult, as well as fetal tissues. C1 is synthesized primarily, if not exclusively, in the gastrointestinal tract (large and small bowel) (43); C2 and C4 by macrophages derived from a variety of organs, including liver, spleen, lung, and bone marrow (44,45); C3 and the C1 inhibitor, by the liver (probably in parenchymal cells) (41,46); and C5 in the liver, the gastrointestinal tract, and possible bone marrow (47,48). The sites of synthesis of C6 to C9 are less firmly established.

Complement Fixation - Probably the most important practical application of complement research has been the development of complement fixation tests for the quantitation of antigens and antibodies. Standard complement fixation tests are performed by mixing dilutions of antigen and antibody in the presence of a known amount of complement. After a suitable incubation period, erythrocytes sensitized with antibody (EA) are added. If complement has not been fixed, the indicator cells (EA) will lyse; fixation by the antigen-antibody complex will lead to a loss of hemolytic complement activity; hence, no lysis of the indicator cells (details given in ref. 49).

$$\text{Antigen (Ag)} + C \longrightarrow + EA \longrightarrow \text{Lysis}$$
$$\text{Antibody (Ab)} + C \longrightarrow + EA \longrightarrow \text{Lysis}$$
$$Ag + Ab + C \longrightarrow AgAbC + EA \longrightarrow \text{No lysis}$$

Recently, a more sensitive complement fixation test that permits detection on a molecular basis of nanogram quantities of antigens or antibodies has been developed (50). This method, the C1 fixation and transfer test, has had useful research applications in the estimation of cell surface antigens and their corresponding antibodies (51). Future applications of this method to clinical problems should be fruitful.

Complement and Human Disease - The complement system plays a central role in the pathogenesis of several human diseases. These clinical conditions can be divided into two general groups: those in which a normal complement system, activated by immune or non-immune mechanisms, leads to damage of the host tissues; and a second group, due to genetic deficiencies of complement biosynthesis. Abundant evidence implicates activation of the complement system in development of the pathological features of systemic lupus erythematosus, rheumatoid arthritis, acute and some chronic forms of glomerulonephritis, serum sickness, and certain autoimmune or drug-induced hemolytic anemias (reviewed in ref. 9). In each case, increased consumption of complement components and/or deposition of complement at sites of tissue damage have been demonstrated. In addition, numerous animal models of these have substantiated the role of complement in the development of characteristic lesions. It should be noted that participation of the complement system is not always accompanied by a change in the concentration of complement in serum. For example, serum complement is normal or even elevated in rheumatoid arthritis, whereas in the joint space consumption of complement and generation of biologically active complement fragments have been clearly demonstrated (52). In lupus erythematosus and acute glomerulonephritis, on the other hand, serum complement is profoundly depressed (53);

and, in fact, changes in complement levels can be used as an index of exacerbation and remission of the disease.

A number of inherited abnormalities of the complement system have been described (54). While generally these abnormalities are qualitatively less severe than most of the other immunodeficiency diseases, several, such as hereditary angioneurotic edema (HANE) (55), and C1r (56) and C5 deficiency disease (30), may have dramatic, even life-threatening, clinical consequences. Hereditary angioneurotic edema (HANE) is a disease in which a genetic deficiency of the natural inhibitor of the first component of complement (C1 INH) leads to attacks of angioedema. Two variants of this disease have been recognized. In the more common form of the disease, the concentration of C1 INH in patients' sera is markedly reduced although the specific activity of the C1 INH that is present is normal. The second and less common form of HANE is characterized by the presence of antigenically intact, but functionally inactive, C1 INH molecules (54). These two forms of the disease cannot be distinguished clinically but are easily identified by the distinctive laboratory findings. In both forms of the disease, during an attack, C1 is activated and the patient develops angioedema probably due to the uninhibited generation of a vasoactive fragment of C2. This disease, therefore, is the result of abnormal regulation of complement activation. A similar circumstance leads to the clinical features of a condition known as congenital hypercatabolism of C3 (57). In this disease, characterized by recurrent severe infections and allergic manifestations, the lack of a naturally occurring enzyme inhibitor (GBGase) leads to increased catabolism of C3. As a result, C3 is not available for normal defense against bacterial infection.

Summary - The chemical isolation and identification of individual complement proteins and the development of sensitive and specific functional measures of complement-dependent biological activities has made it possible over the past few years to advance studies of complement into areas of clinical, genetic, biochemical, and fundamental pathophysiological importance. These advances now permit a logical search for pharmacologic agents that will control complement-dependent disease factors.

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Chapter 22. Immediate Hypersensitivity: Laboratory Models and Experimental Findings

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The general concept of the ability of an animal to fight infection with its immune system is nearly 100 years old, and the role of blood leukocytes and the various cells in lymphoid tissues in this process is well established. It was only in the last 10 years, however, that progress in molecular biology, genetics, cellular biochemistry, and protein chemistry contributed to the unraveling of the multisystem jigsaw puzzle which is immunology.

It has been traditional to refer to two major types of responses as comprising the totality of the immune response. These are the immediate type response and the delayed type response.[1] The names refer to the speed with which an immunologic reaction to a foreign substance is mounted in an animal which had been previously sensitized to that substance. The immediate type response generally occurs within minutes, and the delayed type response takes one to two days to reach its peak. Furthermore, the immediate type response is initiated primarily by circulating antibody molecules (humoral response) while the delayed response involves the infiltration of the affected site by a variety of leukocytes which, in the apparent absence of any demonstrable circulating antibody, are capable of mounting an attack on the offending foreign substance (cellular response). This review is concerned only with reactions of the immediate type. Furthermore, as will be defined below, it is primarily concerned with only one subgroup of the immediate type reactions, namely the models for atopy or "allergy" which are initiated by reagin-like antibodies.

Definitions - To facilitate the discussion, a few of the commonly used terms are listed and defined below:

Adjuvant - A substance which is used in conjunction with an immunogen to augment the immune response or to modify the quantitative distribution of the antibodies of the various immunochemical classes which are produced.

Anaphylaxis - The acute and rapid reaction of an animal having an immediate type of allergy to an antigenic challenge. The reaction can be active if the animal was itself the producer of the responsible antibodies or passive if the antibodies had been administered preformed. Anaphylaxis can furthermore be systemic or local, an example of the latter being passive cutaneous anaphylaxis (PCA).

Antigen - A substance which can combine with an antibody. It is also referred to as an allergen. An immunogen is a substance which induces the production of antibodies. It may, but need not, be an antigen.

Antibodies - Serum proteins which can combine specifically with an antigen. Structurally, antibodies are immunoglobulins and belong to several,

chemically distinguishable classes. These are IgG (Immunoglobulin G), molecular weight 160,000, IgM, molecular weight 900,000, IgA and IgE (or reagin).[2]

Atopy - The immunologic disorders caused by IgE antibodies, colloquially referred to as "allergies." Examples are hay fever, certain forms of asthma and drug hypersensitivities (e.g., "penicillin allergy").

Hapten - A relatively low molecular weight substance which, in itself is not immunogenic or antigenic, can be combined with polymeric carriers such as serum proteins and then induce the production of antibodies specific against itself.

Leukocytes - The white cells of the blood. Among these are lymphocytes, monocytes and polymorphonuclear cells (PMN's). Cells in the last two categories are rich in lysosomes (subcellular organelles containing an assortment of hydrolytic enzymes) and are capable of phagocytosis (i.e., the ingestion of particulate foreign matter). The PMN's are further divided into neutrophils, eosinophils and basophils based on the staining properties of the cytoplasmic granules they contain. Basophils and their relatives, the tissue mast cells, contain in their granules the bulk of the animal's stores of pharmacologic mediators of anaphylaxis (notably histamine and serotonin) and, therefore, play a key role in anaphylaxis while eosinophils also appear to be involved in atopic conditions.

The mechanisms of immediate type hypersensitivity - Aside from the temporal considerations mentioned above, the immediate type reactions can be further categorized by the nature of the antigen and the immunoglobulin class of the antibody initiating the response.[3] Responses initiated by IgG and IgM antibodies usually result in the activation of a series of plasma proteins which act in cascade fashion. These are known as the complement system. While this system will be discussed in detail in the next chapter, it is important to stress here that one consequence of its activation is the production of substances which act as chemotactic agents, attracting the various phagocytic cells to the site where the immune complex is located and, secondarily, bringing about the lysis of the cells to which the complex is attached. In addition, the anaphylotoxins, which are also produced upon the activation of the complement system can affect vascular permeability directly and indirectly. The site of all these events, and the tissue damage which ensues are a function of the localization of the antigen-antibody complex. Since the complexes often accumulate in the kidney, kidney damage is a frequent consequence.

There is a large group of diseases which, while they appear to be immunologically induced, and generally fit into the immediate type of hypersensitivities, nevertheless differ from the classical pattern in that they do not involve the activation of the complement system nor tissue damage. These are the atopic conditions, and their distinguishing feature is that exposure of the organism to the sensitizing allergen results in the direct release of the pharmacologic mediators of anaphylaxis. In addition to histamine and serotonin which are present preformed

in the basophils and the tissue mast cells, several other substances are produced by these cells during anaphylaxis. These are certain prostaglandins,[4] slow-reacting substance of anaphylaxis (SRS-A)[5] and the newly described eosinophil chemotactic factor of anaphylaxis (ECF-A).[6] The structures of neither of the last two substances are known. They both have molecular weights of less than 1000. SRS-A may be an acidic lipid and ECF-A appears to be a peptide. The release of ECF-A during anaphylaxis explains, at least in part, the eosinophilia which generally accompanies atopic conditions, and SRS-A (rather than histamine) may be the major culprit in bringing about asthmatic attacks.

Characteristics of IgE - The physical nature of the immunoglobulin which is responsible for the atopic reactions was an enigma for a long time. It is destroyed by a relatively brief exposure to 56°C,[7] is eluted from sephadex columns at a volume corresponding to a molecular weight of about 190,000, only slightly larger than common IgG. Its concentration in the serum of even highly allergic individuals is so small that it is not possible to demonstrate its presence by the formation of antigen-antibody precipitates. The coincidental discovery of a human patient with a strange myeloma by Johansson and Bennich[8] and the careful separation work of the Ishizaka's[7] finally led to the identification of the agent responsible for atopy as a new class of immunoglobulin which has been termed IgE. In the five years since IgE has been first described, immunoglobulins which are immunochemically and physically similar to IgE have been described in various animal species, including the mouse,[9] rat,[10] rabbit,[11] guinea pig,[12] dog,[13,14] monkey[15] and the cow.[16] In all these species, the characteristics which distinguish IgE (reagin) from all other immunoglobulins are its lability to heat and mercaptoethanol treatment, its presence in trace amounts in the serum, its characteristic fast migration (as a fast γ or slow β band) in immunoelectrophoresis, its ability to bind to and persist in skin sites for very long periods of time, and the complete non-dependence on the complement system for the release of pharmacologic mediators of anaphylaxis upon the interaction of cell-bound IgE with antigen.

The persistence of IgE in the skin has given this immunoglobulin its descriptive name of homocytotropic antibody. It is the basis for its routine assay: Serial dilutions of the antibody solution are injected into the skin of recipient animals. One to three days later, antigen, together with a colloidal dye is injected intravenously. The PCA[17] reaction which results is due to the diffusion of the antigen out of the vasculature, and the combining of the antigen with the tissue-bound antibody molecules causing the release of the pharmacologic mediators from the tissue mast cells. The mediators cause increased vascular permeability, rendering the capillaries in the vicinity of the reaction more permeable to the colloidal dye so that a colored spot results. The size and intensity of the colored spot can be quantitatively related to the concentration of antigen-specific IgE which was present in the injected serum. While the reaction is generally carried out in homologous animals in keeping with the homocytotropic name of the antibody, it has been possible to assay human reagin in monkeys,[18,19] and

mouse reagin in rats.[18,19] Recently, there have been reports[21] that it is even possible to assay human reagin with rat mast cells in an in vitro analog of the PCA reaction. In fact, a serologic homology between human and rat IgE has been demonstrated.[22] When dealing with a heterocytotropic antibody, however, it is essential to be wary of complications which can be caused by the much tighter binding of certain other immunoglobulins to skin sites in the heterologous species than in the homologous species.

While most atopic conditions in man can be explained on the basis of hypersensitivity involving IgE, evidence has been obtained that under certain conditions certain subclasses of IgG may also be homocytotropic.[23,24] The analogous situation in rodents is well established. Here one class of IgG in the mouse,[25] guinea pig[26] and rat[27] is capable of causing a PCA reaction and of causing the antigen-dependent release of pharmacologic mediators from mast cells without the involvement of the complement system. The implications of this dual mechanism remain the subject of much interest.

Production of reaginic antibodies - A number of principles have evolved in recent years to help achieve conditions for evoking an IgE response in laboratory animals. In ordinary immunization schedules IgE, if it is produced at all, is produced in a transient manner early in the immunization. In rats and mice titers are generally low and attempts to boost them by reimmunization fail. An exception is the response of all species tested to infection with live intestinal parasites such as ascaris[28] or the nematode Nippostrongylus brasiliensis (in the rat).[29] Infection with this agent at critical times after immunization with an unrelated protein antigen also can act as an adjuvant for the production of high titers of IgE antibody which is specific for the protein antigen.[30] The use of the proper adjuvant is critical: The use of aluminum hydroxide gels to adsorb the antigen is useful for the immunization of rabbits,[31] mice[32] and guinea pigs,[33] while in rats a specially prepared B. pertussis vaccine consisting of heat-killed Bordetella pertussis organisms is apparently essential.[34]

B. B. Levine and his collaborators have reported that repeated immunization with exceedingly small doses of protein antigens adsorbed to aluminum hydroxide gel produced quite high titers of IgE antibody in mice and guinea pigs.[32,33] Large differences were seen between the responses of various inbred lines of mice to the same haptens when the haptens were coupled to different carrier proteins. The ability to respond to a given protein carrier appears to be linked to the genes controlling histocompatibility (transplantation) antigens.[32] In this respect, as well as in the low doses of antigen which are required for immunization, this system appears to be a good model for the study of the conditions which control reagin production in man. Differences in the response to a hapten when it is coupled to different carrier proteins have also been reported in rats.[28,35]

Tada and his colleagues[36,37,38] as well as others[28] have presented evidence that treatments which ordinarily result in inhibiting the immune

response, such as thymectomy, splenectomy, X-irradiation or treatment with immunosuppressive drugs (cyclophosphamide, actinomycin D, or 5-bromodeoxyuridine) all stimulated production of IgE. Similar observations have been made by Dr. G. J. White of these laboratories[39] using horse anti-rat thymocyte serum as the immunosuppressant. Thus, IgE synthesis appears to be normally inhibited by some other component of the immune response, the production of which is, in turn, inhibited by the immunosuppressive regimens. The thymus derived lymphocytes (T cells) have been shown[40] to play this role.

Methods for studying sensitization and mediator release - The term "sensitization" is used for convenience to denote the process which renders cells capable of releasing histamine upon challenge with antigen. A number of experimental systems are available in which the release of mediators as a consequence of sensitization with reagin and challenge with antigen can be studied both in vivo and in vitro. In addition to the PCA reaction which has already been described, it is possible to quantitate the release of mediators in a unique "in vivo test tube" arrangement by injecting the antibody into the peritoneal cavity and some time later injecting the antigen into the same space.[41] The interaction of antigen with the antibody which is bound to the numerous mast cells in the peritoneal cavity causes these cells to release their pharmacologic mediators of anaphylaxis. The peritoneal washings can be removed, freed of cells, and the supernatant fluid can be assayed for its content of mediators. Other in vivo systems employ anaphylactic collapse or death as their end point. Most of these require the use of actively immunized animals. Aside from the crudeness of the assay end point, these systems introduce further variation caused by animal to animal differences in the degree of immunologic responsiveness, in addition to the variation in the amount of mediator released, and in the systemic sensitivity to the action of the mediators.

In vitro methods for studying cell sensitization and release of pharmacologic mediators offer much better control over animal to animal variation. The tendency of mast cells to degranulate non-specifically in vitro, thus releasing their pharmacologic mediators, requires that a number of experimental variables be meticulously controlled, however. Peritoneal cells from rats[42] and mice[9] have been used. In agreement with the firm binding of IgE to mast cells in skin in vivo where it may persist for periods of several weeks,[43] this antibody binds firmly to the cells in vitro and can cause the release of mediators from the cells even after the cells have been washed free of non-adhering antibody. By contrast, homocytotropic antibodies of the IgG class are much less firmly bound, and do not persist on the cells even after a single washing.[44,45] It is of interest that despite this difference in the avidity of the attachment of the two classes of homocytotropic antibodies to the cells, they can compete with one another for binding to the cells.[44,46] This observation supports the hypothesis that there is a specific receptor for these immunoglobulins on the mast cells. The chemical nature of this receptor is of considerable interest, and the elucidation of its structural features may point the way to the formulation of specific inhibitors of

the attachment of reagins to their receptors.

In vitro methods for studying sensitization and mediator release have also been developed using rabbit leukocytes.[47,48,49] Here matters are somewhat more complex in that the bulk of the histamine in rabbit blood is actually found in the platelets and not the basophils. Challenge of sensitized basophils with antigen results in the release of a soluble factor which, in turn, causes the release of histamine from the platelets. Finally, human leukocytes from non-atopic individuals can be sensitized in vitro with sera from atopic individuals,[50] and histamine can be released upon challenge of the sensitized cells with antigen.

The in vitro system which is the closest model available for studying mediator release in asthma involves the sensitization of chopped lung fragments (from guinea pigs, monkeys or humans)[20,51] with reaginic sera, and collecting the pharmacologic mediators which are released upon challenge of the tissue fragments with antigen. Not only is this the only in vitro system in which the release of SRS-A along with histamine has been described, but it can be shown that it is inhibitable by realistic concentrations of all the drugs which are active in the laboratory and in the clinic in the treatment of asthma.

Sensitive methods are available for the quantitation of the amounts of the various pharmacologic mediators of anaphylaxis which are released during anaphylactic challenge:

1. Histamine can be determined by a fluorometric procedure.[52,53] Alternatively, it can be estimated by a bioassay which depends on measuring the contraction of a piece of guinea pig ileum.[27] The latter procedure is better adapted to handling large numbers of samples although it is not as specific as the fluorometric procedure.

2. SRS-A is also assayed by measuring the contraction of the guinea pig ileum. For this purpose, the muscle is treated with an antihistamine to block its response to histamine.

3. ECF-A is estimated[6] by its chemotactic activity for guinea pig eosinophils. The assay depends on determining the relative rate at which eosinophils penetrate a Millipore® membrane separating a compartment containing mixed leukocytes and a compartment containing a presumed source of ECF-A.

In considering the magnitude of the mediator releasing response, there is, unfortunately, no way of estimating what the maximum amount of SRS-A or ECF-A which might be released might be since neither of these materials is demonstrable in the tissue prior to challenge. In the case of histamine, on the other hand, it is possible under optimal conditions to bring about the release of virtually all of the 30-40 picograms of this mediator which are present in an average rat peritoneal mast cell.[54]

Electron microscopic and fluorescence microscopic studies have shown that the localized application of an antigen to limited regions on the surface of a mast cell result in a similarly localized release of histamine.[55,56] This is most readily shown by use of the releasing agent, compound 48/80, a polymeric amine which can mimic the reagin-mediated histamine release reaction. In both cases the release of mediator occurs within seconds and is not a cytotoxic reaction in that it is not accompanied by the release of other soluble components from the cells, such as potassium ion or soluble enzymes. The antigen concentration which is required for optimal release of histamine is characteristically very low--of the order of a few tenths of a microgram per ml.[57] While inhibition can be achieved when excess antigen is used, the dose response curves tend to be much less steep than the usual antigen-antibody precipitation curves.

Desensitization - The release of histamine from mast cells is an energy-requiring process. In the case of human leukocytes, 2-deoxyglucose, an inhibitor of glucose respiration, inhibits the release of histamine.[58] Similarly, there is no release when the antigen is added to cells in the cold. The first step in the release reaction also requires calcium ions,[59] and is dissociable from the second, respiratory energy-requiring step. While histamine release from rat peritoneal cells is also prevented in the cold, it has not been possible to demonstrate a calcium requirement for that reaction.[44] In both systems the capacity to release histamine once antigen has been added decays rapidly whether or not the release of histamine actually occurs. Thus, when cells are reacted with antigen under conditions which do not permit the release of histamine (e.g., in the absence of calcium ion, in the cold, or in the presence of an inhibitor),[60,61] it is found that the reestablishment of conditions which would ordinarily result in the release of histamine no longer brings about this response. The "anergic" state which results from such an abortive antigenic challenge is referred to as a pharmacologic desensitization. While the mechanism of desensitization is not clearly understood, it is apparent that the biochemical sequence of events starting with the reaction of antigen with antibody and culminating with release of histamine must have a time limit built into it such that histamine release stops even when there still is histamine remaining in the cells. Similarly, if something intervenes to prevent histamine release, the timed reaction may proceed nevertheless, and once it has run its course no histamine release is possible anymore until the limiting step in the release mechanism has been "rewound." It is clear that drugs which are capable of causing desensitization are of considerable interest in the control of atopic conditions since their action is independent of the nature of the antigen or pharmacologic mediator causing the disease symptoms.

The role of cyclic adenosine monophosphate, prostaglandins, and catecholamines in the regulation of mediator release - In 1936, long before the role of catecholamines in the control of adenyl cyclase was appreciated, Schild[62] described the inhibition of the release of histamine from guinea pig lung by epinephrine. It remained for Lichtenstein and Margolis,[63] however, to revive interest in this area. Data have now been accumulated

from various laboratories to document the fact that the cyclic AMP system is involved in the control of the release of pharmacologic mediators of anaphylaxis.[51,58,64,65,66] Conditions which increase the level of cyclic AMP in the cells, such as stimulation of adenyl cyclase by catecholamines or inhibition of the cyclic AMP-destroying diesterase by methylated xanthines (such as aminophylline), markedly inhibit the release of mediators. The actions of agents of these two types can be shown to be synergistic. Conversely, the action of the catecholamines can be inhibited by the β-adrenergic blocking agent, propranolol. Alpha-adrenergic agents (such as norepinephrine) combined with β-adrenergic blockade increase the release of mediators. These effects have been demonstrated with chopped lung preparations from guinea pig, monkey and man, and with the rat peritoneal mast cell system. In all these systems, the catecholamines are active at concentrations in the range 10^{-8} - 10^{-10} M. In the human leukocyte system, the catecholamines are only involved in the calcium independent, antigen-requiring phase of the release reaction[59].

The ability of prostaglandins, and especially PGE_1 and PGE_2 to relax bronchial smooth muscle has been recognized for some time.[67,68] It was thus of considerable interest when it was found that these compounds, in concentrations as low as a few nanograms per rat, could inhibit the release of mediators as well.[69] Similar effects have been reported for the human leukocyte system as well.[58]

Drug mode of action studies - In view of the regulatory role for cyclic AMP in the release of pharmacologic mediators, it is of interest to determine if any of the drugs which are being evaluated for the control of atopic disorders might interact with this system. The antifilarial drug, diethylcarbamazine, has been known to inhibit mediator release.[57] By studying the effect of combinations of this drug with catecholamines, propranolol, and methylxanthines, it was shown that diethylcarbamazine apparently acts as an inhibitor of phosphodiesterase.[64] Disodium cromoglycate, by contrast, could not be shown to interact with any of the components of the cyclic AMP system, and it is therefore concluded that it must inhibit mediator release at a different step in the biochemical sequence leading to release.[64] In fact, it has been suggested[70] that this drug inhibits the action of the phospholipase A which has been postulated by Högberg and Uvnäs,[71] to be involved in the release sequence.

Effect of mediators on end organs - As already mentioned, histamine, serotonin, SRS-A and the kinins, all cause the contraction of smooth muscle when they are added to the tissue *in vivo* or *in vitro* in minute concentrations. The action of histamine can be inhibited by antihistamines, such as pyrilamine or chlorophenyramine and that of serotonin by serotonin antagonists such as lysergic acid and its derivatives.[72] Specific inhibitors of SRS-A or bradykinin have not been described, although the action of these may be inhibited by a variety of non-steroidal antiinflammatory drugs.[73] Caution is essential in interpreting results dealing with the inhibition of the action of mediators on smooth muscle. Not only is there a variation in the sensitivity of various organs of the same species to a given mediator, and a variation in sensitivity of

the same organ from different species,[3] but even the inhibition of the action of a given mediator on different smooth muscle preparations can differ markedly from one system to the next.[74] There is considerable difference between species regarding the anatomical site of the primary anaphylactic lesion. In the rat it is the intestine[72] while in the guinea pig and in man it is the lungs, where the contraction of the bronchial smooth muscle ("bronchospasm") causes severe difficulty in breathing. Thus, as long as there is variation in response between various muscle preparations, human bronchial smooth muscle should be the end organ of primary interest.

Just as the release of pharmacologic mediators from mast cells, their action on smooth muscles is controlled in part by the β-adrenergic system. It has been found, however, that certain β-adrenergic agents are relatively more potent in their action on cardiac muscle than they are on bronchial smooth muscle, and vice versa. This has led to the proposal that there are two types of β-adrenergic receptors, β_1 and β_2, the former being representative of the receptors on cardiac muscle while the latter is the receptor found predominantly on bronchial smooth muscle.[75] Yet other subtypes of β receptors may exist, for example, on the mast cells.[51] Efforts are underway in many labs to capitalized on the differences in specificity of β_1 and β_2 receptors,[76,77,78] but it remains to be seen if these differences are sufficient to be therapeutically useful.

Beta-adrenergic blockade and asthma - Of the atopic conditions, asthma is by far the most troublesome. It would be misleading to imply, however, that reagin antibody is the cause of all asthma. Actually, many patients suffering from this disease do not have any demonstrable atopy.[79] Furthermore, even in those asthmatics where such a hypersensitivity can be shown (the so-called "extrinsic" asthmatics), it is often possible to demonstrate a much higher sensitivity of the bronchial muscle to the action of mediators and a lower sensitivity to the inhibitory action of the catecholamines. An additional role of cholinergic stimulation in the etiology of asthma is recognized, but as yet poorly understood. These considerations have led Szentivanyi[79] to postulate that one of the causes of asthma may be a partial β-blockade. Thus, superimposed on the immunologic mechanism of release of pharmacologic mediators and the possibilities of seeking drugs to control these, agents are needed which can reverse this apparent β-blockade. A few animal models for studying such agents have been proposed. One of these stems from the observation that mice which have been treated with B. pertussis vaccine appear to have an increased sensitivity to histamine and otherwise behave as if they were partly β-blocked. A very interesting new model has been described recently, consisting of inbred lines of guinea pigs having high and low bronchial sensitivity to pharmacologic mediators.[80] The supply of these animals is still very short, but it is to be hoped that careful studies of the specific differences between these strains will point the way to a better understanding and eventual control of this aspect of atopic disease.

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Chapter 23. Transition State Analogs as Enzyme Inhibitors

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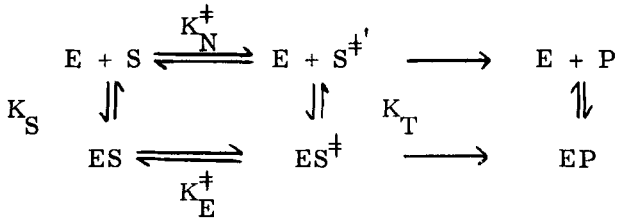
Introduction - This review describes a general approach for the design of specific, potent reversible enzyme inhibitors. The inhibitors that are prepared according to this approach are called transition state analogs. A transition state analog for an enzyme is a compound that resembles (or, in certain special cases, is easily capable of resembling) in structure the substrate portion of the transition state of the enzymatic reaction. The review is divided into two sections, a theoretical section in which the basis for the inhibitory potency of transition state analogs is explained and an experimental section in which the types of transition state analogs that have been prepared are described. A review of this subject by Wolfenden has recently been published.[1]

Theory

Enzymatic Reactions of Single Substrates - Scheme 1 describes a single substrate enzymatic reaction and the corresponding nonenzymatic reaction in terms of the transition state theory of kinetics. E, S, P, ES, EP, $ES^{\ddagger}$, and



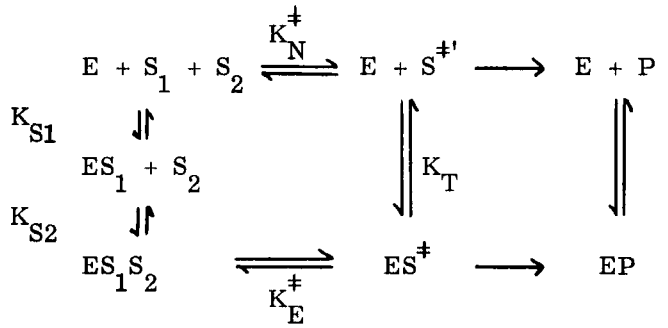
(Scheme 1)

$S^{\ddagger'}$ represent the free enzyme, the substrate, the product, the enzyme-substrate complex, the enzyme-product complex, the transition state of the enzymatic reaction, and the transition state of the corresponding nonenzymatic reaction, respectively. The transition states are defined as the structures of highest energy in the interconversion of S and P and of ES and EP. $K_N^{\ddagger}$ and $K_E^{\ddagger}$ are the equilibrium constants for formation of the transition states of the nonenzymatic and enzymatic reactions, respectively; K_S is the association constant for formation of ES from E and S; and K_T is the association constant for the hypothetical reaction, the binding of $S^{\ddagger'}$ to E. The four equilibrium constants in scheme 1 are related according to the expression $K_T = K_S K_E^{\ddagger}/K_N^{\ddagger}$. Since in transition state theory[2] the equilibrium constant for formation of the transition state is equal to the rate constant of the reaction times the constant h/kT (k is Boltzmann's constant; h is Planck's constant; T is the absolute temperature), this expression becomes $K_T = K_S k_E/k_N$, where k_E is the first-order rate constant for the transformation of the enzyme-substrate complex to enzyme-product complex and k_N is the first-order rate constant for the corresponding nonenzymatic reaction. For a

typical enzymatic reaction the ratio k_E/k_N is 10^{10} or more[1,3]. Thus, the transition state, $S^{\ddagger'}$, is predicted to bind to the enzyme at least 10^{10} times more tightly than the substrate.

This analysis of enzymatic catalysis in terms of transition state theory does not give any information about the degree of similarity between the structure of the substrate portion of $ES^{\ddagger}$ and the structure of $S^{\ddagger'}$. However, it is generally the case that the mechanism of an enzymatic reaction and the mechanism of the corresponding nonenzymatic reaction are similiar in so far as the basic bond-making and bond-breaking steps.[3] Thus, an analog of the substrate portion of $ES^{\ddagger}$ (a transition state analog) is generally also an analog of $S^{\ddagger'}$. Consequently, a transition state analog for such an enzyme should bind to the enzyme much more tightly than the substrate. The fact that the affinity of an enzyme for $S^{\ddagger'}$ is so enormous relative to its affinity for S suggests that even a crude transition state analog should be a potent inhibitor. In addition, it can be shown, by arguments which are given elsewhere,[1,4] that even if the structure of the substrate portion of $ES^{\ddagger}$ and the structure of $S^{\ddagger'}$ are not similar, a transition state analog should still bind to the enzyme much more tightly than the substrate.

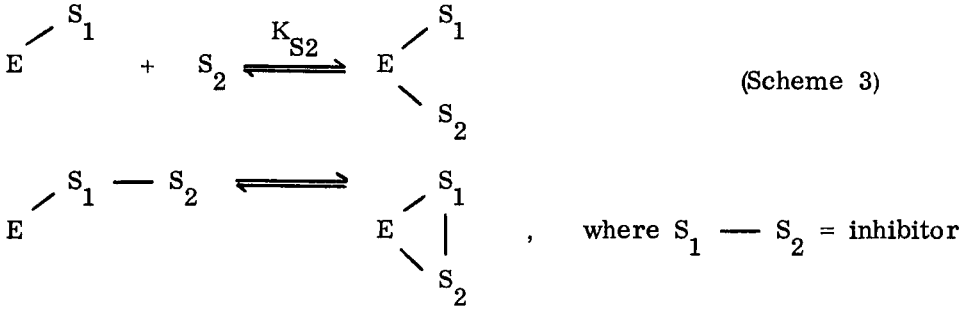
Enzymatic Reactions of Two Substrates That Proceed Via a Ternary Complex - Scheme 2 describes, in terms of transition state theory, an enzymatic reaction between two substrates (S_1, S_2) and the corresponding nonenzymatic reaction. The enzymatic reaction is one that proceeds by a direct reaction between the two substrates that occurs through a ternary complex (ES_1S_2) of E, S_1 and S_2.



(Scheme 2)

In this case $K_T = K_{S1}K_{S2} \cdot K_E^{\ddagger}/K_N^{\ddagger} = K_{S1}K_{S2} \cdot k_E/k_N$. This relationship differs from that based on Scheme 1 only by substitution of a product of association constants for a single association constant and by the dimensions of k_N, which is here a second-order rate constant. Since k_E/k_N is often 10^{10} M or larger and K_{S1} and K_{S2} are typically 10^4 M^{-1} (except in the case of H_2O as a substrate[1]), K_T is immense, and very tight binding of an analog of the substrate portion of $ES^{\ddagger}$ is expected.

It is worth noting here that Scheme 2 shows that potent inhibitors for enzymes that catalyze two substrate reactions are single molecules that incorporate in an appropriate arrangement the primary binding determinants of S_1 and S_2 but lack a functional group that is similar to the structure of the atoms undergoing reaction in the transition state. The association constant for the binding of such inhibitors would be expected to be equal to the product of K_{S1} and K_{S2} times a factor that could be as large as 10^8. This factor is due to the fact that the binding of the S_2 portion of the inhibitor is an intramolecular reaction within an oriented complex, whereas the binding of S_2 itself is intermolecular (Scheme 3).[5,6] These inhibitors may be more similar in structure



to the substrate portion of ES_1S_2 than to that of $ES^{\ddagger}$ and may be called multisubstrate analogs.[1] However, they are also crude transition state analogs, since the initial step in the progression to the transition state of both the enzymatic and nonenzymatic reactions is the coming together of the substrates.

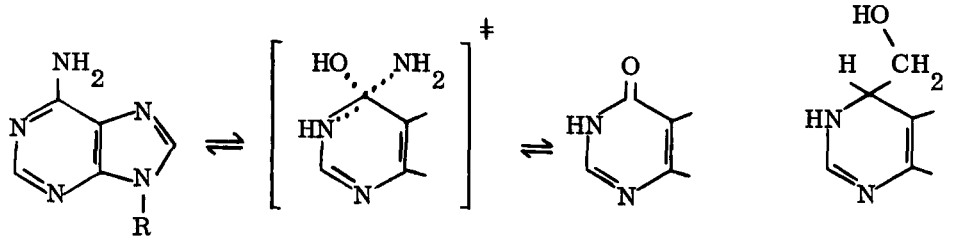
Types of Transition State Analogs

The design of a transition state analog for an enzyme requires a knowledge of the mechanism of the enzymatic reaction or at least an understanding of the possible mechanisms. Fortunately, our knowledge of the mechanisms of enzymatic reactions and of the corresponding nonenzymatic reactions that may be models for the enzymatic ones[3,7] is sufficiently detailed so that the main structural features of the transition state of almost any enzymatic reaction are either known or can be postulated with some confidence. In the design of a transition state analog, it is useful to focus attention upon the metastable intermediate(s), such as a carbanion or carbonium ion, that occurs in the mechanism. An analog of a metastable intermediate is, in fact, a crude transition state analog, since a metastable intermediate and the transition state from which it is formed resemble each other in structure and energy.[8] Because enzymatic reactions fall into classes of reactions with similar mechanisms, a transition state analog for one particular enzymatic reaction should be able to be variously modified to give analogs for the other enzymes in the same mechanistic class. The substituents attached to the functional group that resembles the reacting atoms in the transition state are simply changed to those that satisfy the substrate specificity requirements

of each enzyme within the mechanistic class.

The examples of transition state analogs that are described below have been chosen a) because they appear to resemble the postulated transition state and b) because they appear to bind to the enzyme much more tightly than the substrate(s). It should be emphasized that in most of the examples the structures of the transition state and of the corresponding enzyme-transition state analog complex are reasonable postulates rather than verities established through extensive experimentation. The inhibitory potency of the analogs (P) has been expressed as the ratio of the association constant for binding of the analog to the enzyme to that for binding of the substrate (K_S). In many cases, the value of K_S has not been determined; and the reciprocal of the Michaelis constant, a kinetic parameter whose value is equal to the substrate concentration required for half-maximal velocity of the enzymatic reaction,[9] has been used in its place.

Analogs of tetrahedral transition states - Adenosine deaminase catalyzes the hydrolysis of adenosine (1) to inosine (3), probably via a tetrahedral-like transition state formed by the attack of water (2). 1,6-Dihydro-6-hydroxymethylpurine (4) resembles 2 by virtue of the H on N5, the tetrahedral geometry of C6, and, in one conformation, the position of hydroxyl group. The value of P for one isomer of 4 is 40.[10] The deamination of cytosine

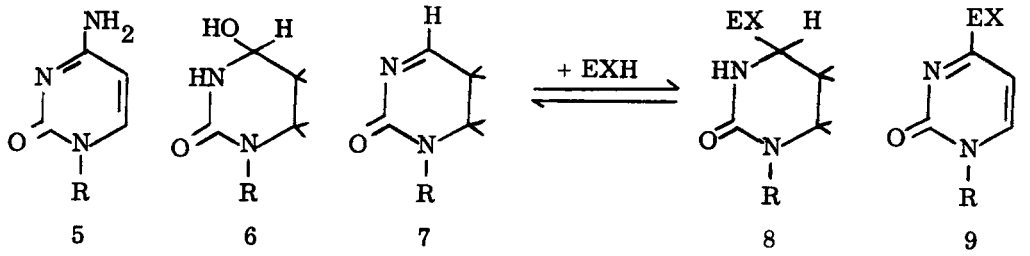


1 2 3 4

R = β-D-ribofuranosyl

nucleosides (5) by enzymes from various sources[11,12] is markedly inhibited by 3,4,5,6-tetrahydrouridine (6), with P for cytidine deaminase from E. coli being 830.[12] Although it is reasonable to hypothesize that the transition state for this reaction is similar to 2 and so accounts for the inhibition by 6, the possibility that a nucleophilic group of the enzyme adds reversibly to the dehydrated form of 6, which may form readily from 6 (7,8),[13] should also be considered. If the enzymatic reaction proceeds by a double displacement via 4-pyrimidinyl enzyme intermediate (9), 8 is analogous to the tetrahedral transition states for the formation and hydrolysis of 9 (see the description of chymotrypsin).

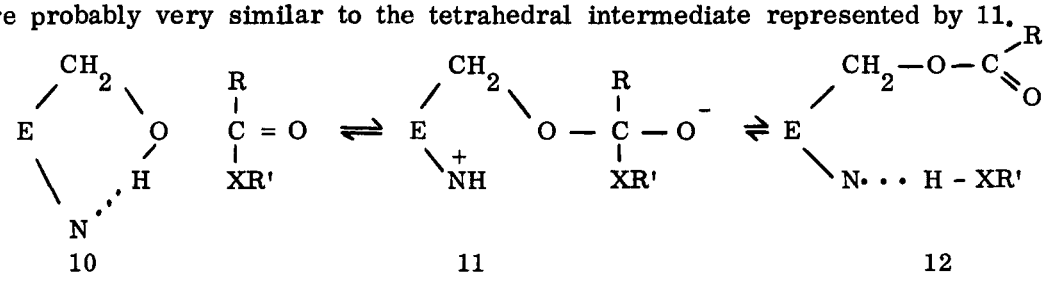
Chymotrypsin is one of a class of enzymes that catalyze the transfer of acyl groups (R—C = O) between various acceptors (XR') by way of a covalent



5 6 7 8 9

R = β-D-ribofuranosyl, EXH = enzyme

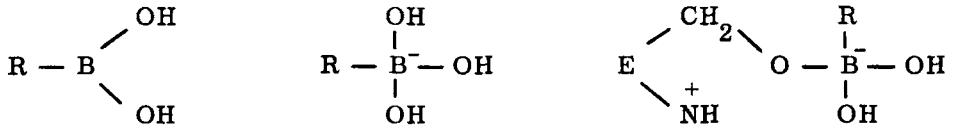
acyl-enzyme intermediate (10-12). In the case of chymotrypsin, the acyl-enzyme (12) is formed from the hydroxyl group of a seryl residue (CH_2OH), with the participation of the imidazole group of a nearby histidine residue (N) in proton transfers.[14] The transition states for the acyl transfer reactions are probably very similar to the tetrahedral intermediate represented by 11.



10 11 12

XR' = OR, OH, NHR

A possible transition state analog for chymotrypsin is 2-phenylethaneboronic acid (13), which is an effective competitive inhibitor of the enzyme (P = 125).[15] Since the trigonal, planar boronic acids are known to ionize in dilute base by the addition of hydroxide ion to form anionic tetrahedral adducts (14), the seryl hydroxyl group of chymotrypsin may add to the boron in the same way to yield a structure (15) similar to 11. The 2-phenylethyl group satisfies the



13 14 15

R = $PhCH_2CH_2$-

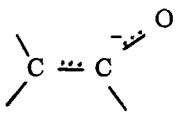
specificity of chymotrypsin for an aromatic side chain.[14,16] Other boronic acids are also inhibitors of chymotrypsin, but they are less effective than 13.[16,17] The structures of the complex that is formed between chymotrypsin and 13 is, by no means, proven to be 15; several other possibilities have been hypothesized.[15-17] It should be pointed out that if 15 is correct, this type of transition state analog is unusual because it forms a covalent bond

with the enzyme and so has a different structure in the complex than in solution. A method for predicting the maximal possible affinity of an enzyme for such an analog is described in reference 15.

Penicillin has been suggested to be a transition state analog as well as a covalent inhibitor of peptidoglycan transpeptidase.[18] The basis of this suggestion is the fact that the β-lactam function of penicillin is not planar. Thus, it is possible for the spatial relationships among the atoms of the tetrahedral intermediate that is formed from the susceptible planar peptide bond in the substrate to be similar to those among the atoms of penicillin. Since it appears that subsequent to the noncovalent interaction between penicillin and the transpeptidase, virtually irreversible acylation of the active site SH function by the lactam group occurs, it will probably be very difficult to determine the association constant for the initial noncovalent binding of penicillin to transpeptidase. Thus, it may not be possible to determine whether the criterion that a transition state analog binds more tightly than the corresponding substrate is satisfied here. In any event, this hypothesis for the action of penicillin illustrates a useful general approach for the design of very specific and potent irreversible enzyme inhibitors, which is to devise structures that are both transition state analogs and aminoacid-modifying reagents.[1]

Other possible analogs, which will only be mentioned here, are L-methionine-(S)-sulfoximine phosphate and L-benzylsuccinate; these compounds bind tightly to glutamine synthetase and carboxypeptidase, respectively, and can resemble to some extent the tetrahedral-like transition states.[19,20]

Analogs of Carbanion-Like Transition States - There is a similarity between the structure of an enolate anion (16) and a carboxylate group (17), and it has been found that several enzymatic reactions in which the transition state probably resembles an enolate anion are strongly inhibited by the analogous carboxylate anion. The most thoroughly studied analog of this type is



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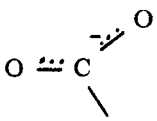

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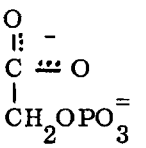

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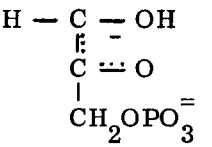

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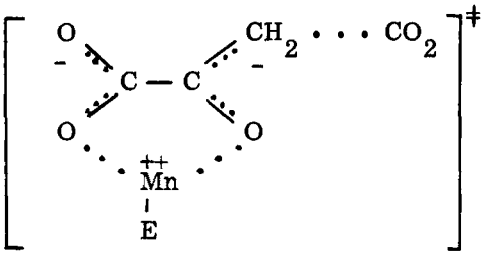
phosphoglycolic acid (18) which resembles the metastable enediolate intermediate (19) that occurs in the interconversion of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate catalyzed by triose phosphate isomerase.[21] The value of P for phosphoglycolate varies with pH; at pH 7 it is 75. Another example of this type may be oxalate (21), which is a potent inhibitor of an Mn^{++}-requiring enzyme (P = 300)[22,23] that decarboxylates oxaloacetate (20). Oxalate is also an effective inhibitor (P = 200) of the Mn-containing enzyme

that catalyzes the transfer of this carboxyl group from oxaloacetate to biotin[24] and may also act here as a transition state analog, although the investigators propose an alternative mechanism for carboxyl transfer.



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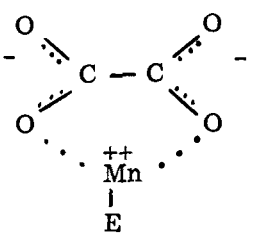
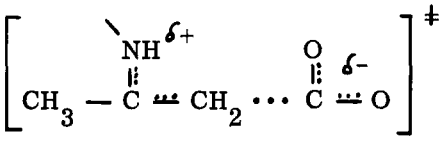


21

The enzyme acetoacetate decarboxylase catalyzes the decarboxylation of acetoacetate by way of a Schiff base formed with the ε-amino group of a lysyl residue of the enzyme (22). Acetylacetone is a potent inhibitor of the enzyme (P = 10,000), probably because it can easily form an enamine with the enzyme (23) that resembles the transition state.[25] Other examples of transition state



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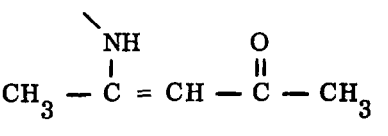
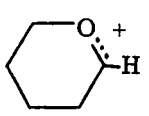


23

analogs for planar, carbanion-like transition states, which are described in detail elsewhere, are pyrrole-2-carboxylate and 17-β-dihydroequilenin; these compounds resemble the transition states that are postulated for the enzymatic racemization of proline and isomerization of Δ^5-3-ketosteroids, respectively.[26]

Analogs of Carbonium-Ion Like Transition States - Most glycosyl-transferring enzymes are strongly inhibited by the δ-lactone (25) analogous to the corresponding substrate (P as large as 10,000).[27-29] The explanation is probably that the transition states for these reactions are similar to alkoxycarbonium ions (24), which like the lactones, have a half-chair conformation and positive charge on the ring oxygen atom and carbon 1 of the ring. Norjirimycin, an antibiotic that differs from glucose only by the substituion of an NH group for



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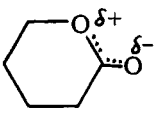

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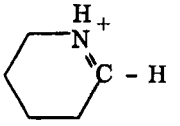

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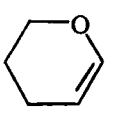
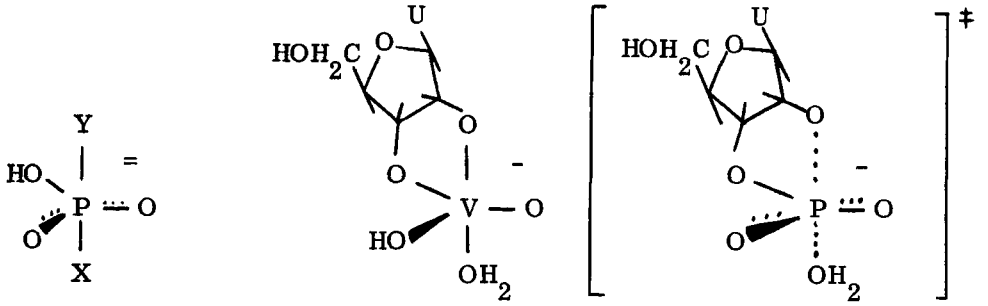


27

oxygen in the ring, can exist in a form that is an even better analog of 24 (26) and has been found to be an even better inhibitor of glucosidases than D-glucono-δ-lactone.[30] It appears that in certain cases a relatively planar conformation of the ring is a sufficient similarity to 24 to allow tight binding,

since β-galactosidases are inhibited (P = 3-160) by D-galactal (27).[31]

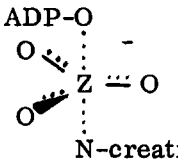
Analogs of Transition States for Phosphoryl Transfer - The transition states for most enzymatic phosphoryl transfer reactions are probably very much like a trigonal bipyramid of pentacovalent phosphorus, with the entering and leaving groups (X, Y) in apical positions (28). A complex between oxovanadium ion (IV) and uridine binds very tightly to the enzyme ribonuclease (P = 1700),



28 29 30

U = uracil

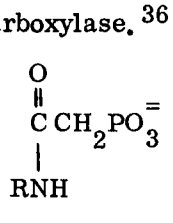
probably because the complex has a structure (29) that mimics the transition state for ribonuclease-catalyzed hydrolysis of uridine-2',3'-cyclic phosphate (30).[4] The enzyme that catalyzes the transfer of a phosphoryl group from adenosine triphosphate to creatine forms a very stable quarternary complex with adenosine diphosphate (ADP), creatine, and nitrate (or similar anions). This case appears to be an example of the organization on the enzyme of a transition-state-like complex from several compounds, which is possible here due to the similarity of NO_3^- and the base of the phosphorus trigonal bipyramid (31).[32]



31, Z = P for the transition state
Z = N for the analog complex

Analogs For Multisubstrate Reactions - N-(Phosphonacetyl)-L-aspartate (32) incorporates features of both substrates of the enzyme aspartate transcarbamylase (33), although it does not contain a tetrahedral functional group analogous to the tetrahedral-like transition state that is expected for this carbamyl transfer reaction (34). The association constant for binding of this compound to aspartate transcarbamylase is very large (4×10^7 M^{-1}).[33] The virtually irreversible inhibition of the enzyme that catalyzes the transfer of an acetyl group between the thiol group of coenzyme A and the hydroxyl group of carnitine by a compound in which coenzyme A and carnitine are appropriately linked together (35) appears to be another example of this type

of analog.[34] Multisubstrate analogs have also been prepared for nicotinamide adenine dinucleotide-dependent enzymes[35] and for ribulose diphosphate carboxylase.[36]



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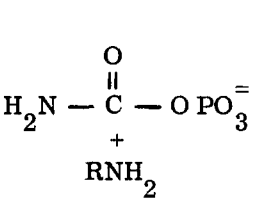

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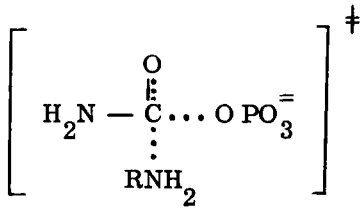
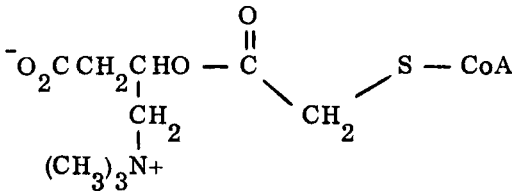


34

R = $-CH(CO_2^-)CH_2CO_2^-$



35, CoA = coenzyme A

Conclusion - There are many other strong inhibitors of enzymes that have not been discussed here. The explanation for the effectiveness of some of these compounds will almost certainly be found in their resemblance to the transition states of the enzymatic reactions that they inhibit. The application of the transition state theory of kinetics to enzymatic catalysis has provided both an explanation of why many potent inhibitors of enzymes are potent inhibitors and a basis for the design of new potent inhibitors.

Acknowledgment - The author is indebted to Dr. Kim Collins for his aid in searching the literature.

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Section 6 - Topics in Chemistry

Editor: Robert A. Wiley, University of Kansas, Lawrence, Kansas

Chapter 24. Biopharmaceutics and Pharmacokinetics

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University of California, San Francisco

Biopharmaceutics - The relationship between the physicochemical properties of a drug in a dosage form and the biological response observed following its administration is the subject of biopharmaceutical studies. The ability to carry out in vitro studies on the dosage form, which show in vivo correlations with biological availability is a fundamental problem and has resulted in an excellent "Guide for Biopharmaceutical Studies in Man" prepared by a committee of the APhA Academy of Pharmaceutical Sciences[1]. In addition, three texts[2-4] have been published in the last year dealing with biopharmaceutics and related pharmacokinetics. In vitro dissolution studies were shown to correlate with in vivo availability measurements for commercial acetaminophen tablets[5], two different timed release nitroglycerin tablets[6-7], different formulations of sulphadimidine tablets[8], and five different crystalline phases of fluprednisolone[9].

Wagner et al.[10] demonstrated a strong correlation between in vitro dissolution studies and in vivo blood levels following oral dosing of four different commercial brands of warfarin tablets. There were marked intersubject and intrasubject variations in the terminal half-lives of warfarin, which required the authors to make some assumptions concerning either constant clearance or constant volumes of distribution from study to study when relative availabilities were determined, since no I.V. doses were given the patients in this report. The authors chose to assume that the volume of distribution was constant and although relative availabilities of the four dosage forms would be maintained if constant clearance were assumed, this problem should receive attention in future studies when varying half-lives are observed. Workers at the Food and Drug Directorate of Canada published a series of articles pointing out the inadequacy of using a single dissolution test to predict relative bioavailability for an entire series of commercial products. Studies dealt with sulfadiazine[11], phenylbutazone[12,13] and nitrofurantoin[14].

Chiou and Riegelman[15] reviewed the pharmaceutical applications of solid dispersion systems. These authors found complete and rapid absorption of griseofulvin dispersed in polyethylene glycol 6000 for both capsule and tablet dosage forms, in contrast to irregular and incomplete absorption from commercially available micronized drug.[16] Munzel[17] reviewed various formulation variables which can influence drug action. Kakemi and co-workers[18] studied the effects of buffer components, osmotic

pressure, injection volume, buffercapacity and pH of formulated intramuscular injections. Low osmotic pressure and low pH solutions yielded decreased absorption of nonionized drugs due to morphological changes in the muscle. Miller and Fincher[19] demonstrated an inverse correlation between area under the blood level time curve and the particle size of phenobarbital in intramuscular suspensions given to beagle dogs. Cressman and Sumner[20] have shown that purebred beagle dogs may serve as a good model for evaluating the bioavailability of aminorex fumarate from sustained-release tablets. Hanselmann and Voigt[21] reviewed the literature on prolonged action drugs. White et al.[22] showed that oral administration of digoxin with and without meals had little effect on the bioavailability of the drug. Although the areas under the plasma curves for the first eight hours appear different, the drug has such a long half-life that when total areas to infinite time are compared, no difference would be expected, as was confirmed in a four week multiple dosing study. Lindenbaum et al[23] found marked variances in the blood levels of digoxin during the first five hours after oral administration of four commercial products. Unfortunately they did not take blood samples beyond five hours and therefore it is possible that no differences in biologic availability between the products exist. Since the study concerns a drug with a long half-life which is given repeatedly, availability of the drug is the parameter which is important clinically, not initial blood levels. The study is poorly designed since availability can only be inferred by assuming absorption is complete in five hours.

Physiologic factors and drug interactions may also affect the extent of drug absorption. Nightingale and co-workers[24] found that stimulation of bile flow in the rat increased the gastrointestinal absorption of sulfadiazine, while Winne[25] described two different four compartment models which relate intestinal absorption to blood flow. The relative amount of drug absorption from the stomach was shown to be negligible compared to intestinal absorption for digitoxin[26], sulfamethoxazole[27], warfarin[28,29], cephaloridine and cephalothin[30]. Kojima et al.[31] found that the presence of food decreased the pharmacological activity of phenobarbital by decreasing the rate of absorption in the rat and that this decreased absorption rate is due primarily to slowed gastric emptying. Bianchine et al.[32] correlated the pharmacologic effect of L-dopa to its blood levels and pointed out the marked effects of stomach emptying on the rate and extent of availability of this drug. In addition, a number of drug-drug interactions may be explained as a result of altered stomach emptying rates which can in turn influence the rate and extent of bioavailability. Hayton and Levy[33] showed that SKF 525A decreased the absorption rate of orally administered sulfacetamide by inhibiting stomach emptying. It is suggested that the tricyclic anti-depressants delay oxyphenbutazone absorption[34] by the same process, while barbital probably increases the absorption rate of aminopyrine in the rabbit by increasing the stomach emptying rate.[35]

Antacids are a class of over-the-counter drugs which might markedly affect the bioavailability of another drug. For example, Hurwitz and

Sheehan[36] showed that although co-administration of antacids containing Al ion slowed the rate of pentobarbital absorption and delayed its pharmacologic effect, the total amount of drug absorbed was unaffected by the antacid. Hurwitz[37] also studied the effects of antacids on sulfadiazine and quinine, noting changes in stomach emptying time and solubility. Barr et al.[38] demonstrated the decreased availability (approx. 50%) of tetracycline from commercial capsules when 2 g. of sodium bicarbonate is given at the same time. When tetracycline was dissolved prior to administration, no differences in availability were observed with or without concomitant administration of sodium bicarbonate, indicating that the dissolution rate step is involved in the decreased absorption of tetracycline capsules. Robinson et al.[39] found that concomitant administration of a bulk forming colloid, psyllium hydrophyllic mucilloid or an antacid with warfarin, decreased the availability of the drug, while cholestyramine decreased warfarin blood levels apparently by binding the drug and decreasing absorption.

Since the majority of drugs are given orally, much work has been directed toward understanding the mechanisms of intestinal absorption and attempting to modify the drug or dosage form so as to improve absorption. Taraszka[40] found that the transfer rate across the in vitro rat intestine for a clindamycin analogue could be fairly well predicted by assuming that only unionized molecules of the drug were transferred and by correcting clindamycin transfer rates for the difference in pK_a of the analogue. However, Crouthamel et al.[41] reported significant transport of ionized sulfaethidole and barbital from the rat intestine, and very reduced absorption of drug from the stomach at pH values ranging from 3.5 to 8.3. Benet et al.[42] and Morishita and co-workers[43] studied the effect of buffer components on the absorption of drugs, while Mayersohn and co-workers studied the influence of various sugars[44], hypotonic and hypertonic solutions[45], and high potassium ion concentration[46] on passive drug transfer across the everted rat intestine. This work and the study of Nayak and Benet[47] on drug transfer across rat intestinal musculature after EDTA treatment are consistent with a proposed mechanism whereby polar compounds are assumed to traverse the isolated, everted rat intestine via intercellular channels existing between adjacent mucosal epithelium cells.

Drug transport and availability have also been measured for routes of administration other than oral. Rectal absorption of acetaminophen[48] and salicylate[49], buccal absorption of barbituates[50] and a series of n-alkanoic acids[51], peritoneal dialysis[52-54], penetration across the vitreous barrier of the eye[55], and the significance of vehicle composition in the skin penetration of fluocinolone acetonide[56,57] were all studied. Lymphatic absorption of digitoxin, digoxin[58] and iopanoic acid[59] was found to be insignificant. Lucas et al.[60] experimentally confirmed that compounds administered by intraperitoneal injection were absorbed primarily through the portal circulation, while Bederka and co-workers[61] found that absorption of drug into the circulation following an i.m. injection was much more sensitive to changes in blood flow to the muscle than to molecular weight of the absorbed species.

Pharmacokinetics - While biopharmaceutics is essentially the study of the effects of the dosage form on the input (absorption) of a drug into a biological system, pharmacokinetics is concerned with the disposition of the drug once it becomes available for input. The goal of a pharmacokinetic study should be to relate a clinical response to the pharmacokinetic parameters used in describing the time course of a drug and its metabolites in the body. Studies relating pharmacologic effect to pharmacokinetic parameters have appeared for ethanol[62], fenclozic acid[63], disopyramide phosphate[64], and heparin[65]. Gibaldi et al.[66] presented a very well written analysis of the relation between pharmacologic effects and drug distribution in multicompartment systems. Jusko[67] showed that the necrobiotic response of chemotherapeutic agents which attach irreversibly to cell receptors may be quantitated by log effect vs. dose plots if one assumes that very little drug is actually lost by irreversible binding. In essence he has approximated the irreversible nature of these agents by assuming that they appear to interact reversibly. Smolen and Schoenwald[68] used the mydriatic response of tropicamide in rabbits, a pharmacologic measurement, to measure the absorption rate of the drug into the central systemic compartment. The validity of the method is tested using known i.v. infusion rates of the drug. Transcorneal absorption was also measured[69]. Although the method appears impressive, it is unfortunate that direct verification of the results was not made, at least once, by measuring plasma concentrations. Smolen[70] also used pharmacologic data to determine the bioavailability and kinetic model for tridihexethyl chloride following ophthalmic and oral administration of the drug. Although there are minor errors in modelling and computer fitting of the data, the principles brought forth are important contributions.

Most work during the past year involved use of the more conventional compartment models which have been described in a number of books[2,3,71] and articles.[72-74] However, a few investigators described solutions for nonlinear pharmacokinetic equations.[2,75,76] Westlake[77] points out that even though the least-squares estimates of pharmacokinetic model parameters might be considerably in error, they will probably enable reasonably accurate predictions of blood levels to be made. However, considerable disparity will exist between exact tissue drug levels and predicted levels based on least-squares estimates. Although this is true within the context presented, the author should have also pointed out that there can be no exact tissue levels, since the amount of drug in an unsampled distribution compartment is model dependent and only results as a function of how the author defines elimination from the system. This last comment is also appropriate to the work of Noordhoek[78] who failed to find predicted tissue levels of hexobarbital in mice. Gibaldi and Weintraub[79] pointed out that premature termination of single dose pharmacokinetic studies due to poor sampling design or to analytical methods which are not sufficiently sensitive, may yield erroneous underestimates of half-lives. This could lead to gross errors in the estimates of steady-state blood levels or to the invalid assumption that dose dependent kinetics were observed following multiple dosing.

During the past year more than 100 papers have appeared describing the pharmacokinetics-the absorption, distribution, and metabolism of various drugs. Although many of these papers cannot be covered in this limited review, the importance of these studies should not be minimized, since over half of these studies were carried out in man, and serve as a comprehensive data base for the use of pharmacokinetic data in the development of rational dosing regimens[80]. Human studies following i.v. administration of phosphonomycin[81] and cyclophosphamide[82] yielded good data consistent with a two compartment model. Data on toxogonin[83] and p-chlorbenzolsulfone[84] would probably yield better fits if a two compartment model was utilized. Mark et al.[85] used a whole-body counter to follow the uptake, distribution and excretion of a single breath of halothane-^{82}Br. A two-compartment model was used to fit the resulting data. Bischoff et al.[86] expanded their preliminary model of methotrexate pharmacokinetics by including multicompartment representations for biliary excretion and movement of the drug through the g.i. tract with partial reabsorption. The new model is consistent with data from the mouse, rat, dog, monkey and man at several doses. Doluisio et al.[87] showed that following i.m. injection of ampicillin trihydrate suspensions or a dicloxacillin sodium solution, the slope of the terminal phase of the serum level - time curves corresponded to the absorption rate of the drug from the i.m. injection site rather than to the elimination rate of the drug from the body. Without i.v. data for these compounds erroneous results as to absorption and elimination rates would have resulted. These authors also pointed out that i.m. injections of the above drugs as well as ampicillin sodium solutions resulted in decreased availability of the drug (65-75%) as compared to i.v. injections.

A number of investigators noted decreased availability of drugs subject to hepatic metabolism when these drugs were administered orally as compared to i.v. dosing. The influence of route of administration on availability was noted for oral doses of lidocaine,[88,89] oral propranolol[90-92], inhaled isoproterenol[90,91], intraperitoneal injections of marihuana[93], and oral helveticosal acetonide[94]. Gibaldi et al.[95] developed equations which would predict the fraction of drug metabolized on the first pass through the liver following oral administration if i.v. areas under the curve were known. The application to propranolol data was incomplete, however, since although they used blood flow rates, the areas under the curve were from plasma data, and would only yield valid estimates if the partition coefficient for propranolol between plasma and red blood cells were unity. Alvarez[96] pointed out that although in vivo half-lives of pentobarbital and thiopental differed from those found in the isolated perfused rat liver, clearance measurements were similar. Ohnhaus et al.[97] noted changes in liver blood flow during enzyme induction.

The effects of biliary excretion on drug kinetics is receiving increasing attention in pharmacokinetic studies. Specific studies on the biliary excretion of nitrofurantoin[98], barbiturates[99], a series of penicillins[100], and demethylchlortetracycline[101] have been reported.

Phenobarbital was found to increase biliary flow in rats[102]. This increase is due to enhanced formation of the bile salt-independent fraction of canalicular bile production. Clark et al.[103] showed that low molecular weight anionic drugs administered to rats by retrograde biliary infusion are excreted primarily in the urine indicating absorption from the biliary tract. High molecular weight anionic drugs, in contrast, were excreted predominantly in the bile.

The protein binding of diphenylhydantoin was found to decrease in uremic patients[104]. The free metabolite levels of this drug, estimated on the basis of in vitro binding studies, were found to correlate with the in vivo incidence of diphenylhydantoin-induced gingival hyperplasia in man and rat.[105] Drug interactions involving displacement from proteins were investigated for phenylbutazone and sulphadoxine[106] and for warfarin and a series of acidic drugs.[107] McArthur et al.[108] showed that salicylic acid and phenobarbital bind to human red cells as well as plasma proteins and that calculations as to free drug concentration in vivo will be incorrect if they are based only on in vitro plasma protein binding studies. These same authors[109] suggest that the antirheumatic drugs may act by binding to serum proteins and thereby increase the proportion of free peptides in the blood by competitive protein binding of the drugs.

Drug-drug interactions in man received considerable study during the past year. Levy[110] reviewed those aspects of drug biotransformation interactions in man that are seen with administration of the nonnarcotic analgesics, while Dayton and Perel[111] presented a more general review of the mechanisms involved in drug interactions. Levy and co-workers found no inhibition in glucuronide and sulfate formation when acetaminophen and salicylic acid were given concomitantly as compared to separate administration of each drug. These studies[112,113] led to the conclusion that of the several combinations of acetaminophen, salicylate, and salicylamide studied, salicylamide is the major determinant in the biotransformation interactions encountered. The effect of metabolic enzyme induction was also studied for the following inducer-drug pairs: disulfuram-antipyrine [114], phenobarbital-glyceryl guaiacolate ether[115], imipramine and desimipramine-barbiturates[116,117], diphenylhydantoin-phenobarbital[118] and hexobarbital[119], hypnotic drugs-warfarin[120], chloral hydrate-coumarin anticoagulants[121], phenylbutazone and diphenylhydantoin-digitoxin[122], carbamazepine-diphenylhydantoin and warfarin.[123] References 115,118 and 120-123 report increased metabolic rates for the drug as a function of the inducer. The remaining reported interactions resulted in decreased metabolic rates. Other drug-drug interactions appeared to influence distribution and membrane transport. Insulin altered the tissue distribution of tolbutamide, but not chlorpropamide.[124] Ethanol was found to increase the in vivo lipid solubility of phenobarbital and its rate of transport across the peritoneum and uptake into the blood, and therefore a corresponding uptake into the brain.[125] The effect was much less pronounced on pentobarbital uptake because of its inherent high lipid solubility and rapid absorption. Increasing interest in differentiating genetic and environmental influences on drug disposition, is reflected in a

number of publications this past year.[126] Specific studies have dealt with halothane metabolism[127,128], the pharmacokinetics of monomethylated tricyclic antidepressants[129], and ethanol metabolism.[130]

Davies and co-workers[131] demonstrated the use of antipyrine half-life as a measure of an individual's capacity to metabolize phenylbutazone and oxyphenbutazone. Shibasaki et al.[132] followed acetaminophen and its glucuronide metabolite in the blood and urine of rabbits. They found that the rate limiting step for appearance of this drug in the urine was the excretion of the metabolite rather than the metabolite formation as had previously been assumed. Since a simple A→B→C model was not found to hold, a catenary chain model with an additional compartment which may be partially bipassed was introduced. Although good fits of the data are obtained, the model has no biological meaning since it suggests a significant amount of an intermediate metabolite. Fleischmann[133] noted that metampicillin administered intramuscularly or intraveneously does not undergo metabolic changes in the blood or bile, but rather in the kidney. Findings of significant kidney metabolism in this work and other preliminary reports suggest that this factor must be adequately identified when comparisons of dosage form availability by different routes of administration are made.

Structure activity relationships between physicochemical properties of a drug and its pharmacokinetic disposition have been investigated. Seydel and Wempe[134] prepared a sequence of new highly active sulfapyridines on the basis of predicted correlations of pharmacokinetic behavior in physiological systems with certain physicochemical properties. Johnson and Maibach[135] found that partition coefficient, in addition to pK, is important in determining the rate of excretion of drugs in human eccrine sweat. Beckett and Brookes[136] orally co-administered a number of amphetamine type drugs and followed the urinary excretion of the unchanged drug using g.l.c. analyses. They found that halogen substitution caused a marked decrease in total unchanged drug excretion as well as a delayed peak excretion time. Substitution of a second methyl group on the alpha carbon atom caused a significant increase in total unchanged drug excretion without affecting the peak excretion time.

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Chapter 25. Reactions of Interest in Medicinal Chemistry

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The stepped up abstracting of useful synthetic procedures from the international chemical literature by the journal Synthesis is noted (1844 abstracts to Dec. 1971) as well as the appearance (in soft cover) of the first of the Annual Reports in Organic Synthesis, edited by J. McMurry and R.B. Miller. To avoid possible overlap with material in other chapters, specialized reactions for steroids and amino acids have not been included here; and, as in previous years, the materials actually presented are necessarily highly selective.

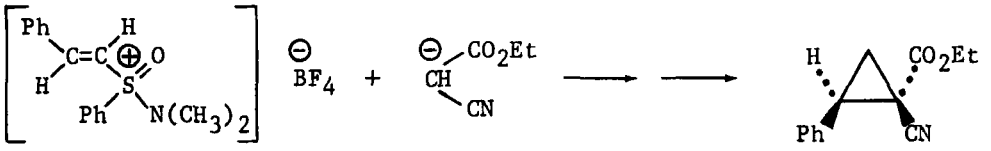
Useful Reagents (not elsewhere classified) - A stable (several months under N_2), storable hydroborating and reducing agent which is liquid at room temperature is provided by dimethylsulfide-borane.[1] An excellent reagent for the oxidation of a) Schiff bases to *oxaziridines*, b) nitrogen-containing aromatic heterocycles to *N-oxides*, and c) nitrosamines to *nitramines* is t-amylhydroperoxide with $MoCl_5$.[2] $Tl(NO_3)_3$ is useful for a) the degradation of monoalkylacetylenes to carboxylic acids containing one less carbon atom, b) synthesis of *acyloins* from dialkylacetylenes, and c) synthesis of *methyl arylacetates* from acetophenones.[3,4]

An extremely simple procedure for the preparation of *esters*, *acetals* or *ketals* at room temperature involves the use of a dehydrated sulfonated polystyrene copolymer together with a drying agent ($CaSO_4$).[5] On a preparative scale, 1,4-dioxaspiro[4.5]decane was produced in 90% yield from cyclohexanone and ethylene glycol. Somewhat analogously, *ketimines* and *enamines* can be prepared in high yield by the reaction of the appropriate ketones and amines in benzene or ether at room temperature in the presence of molecular sieves.[6] This general method has been successful even with medium-sized ring ketones and camphor, which are rather hindered. Sodium borohydride on silica gel reduces oximes to the corresponding *hydroxylamines* in benzene at room temperature.[7]

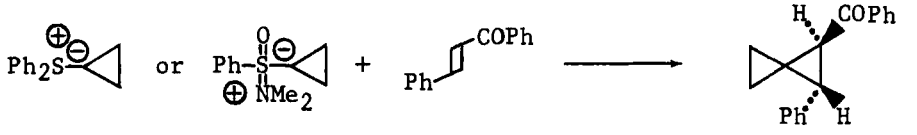
Protective Groups and Their Removal - The *acetylenic linkage* in compounds containing both double and triple bonds can be protected as the acetylene-dicobalt hexacarbonyl complex by treatment with cobalt carbonyl.[8] After reaction at the non-coordinated olefinic bond is completed, the acetylene may be regenerated with $Fe(NO_3)_3 \cdot 9H_2O$. *Methylene groups* adjacent to the carbonyl in carbonyl compounds may be protected as dithiolanes or dithianes by condensation with ethylene or trimethylene dithiotosylate, or for unreactive methylenes, by initial activation to an enamine or a hydroxymethylene derivative followed by condensation.[9] The methylene groups may be regenerated by Raney Ni reduction or may be converted to a carbonyl function by mercuric ion catalyzed hydrolysis. Oxidative hydrolysis appears to be a superior method for removal of the dithioketal groups,

especially when oxygenated or olefinic functions are present. Thus, N-bromosuccinimide or N-chlorosuccinimide-$AgNO_3$ in aqueous CH_3CN or Me_2CO readily converts this group to a ketone at room temperature or below.[10] Chloramine-T in H_2O or MeOH has also been used successfully to oxidatively cleave 1,3-dithiolanes[11] and 1,3-oxathiolanes.[12]

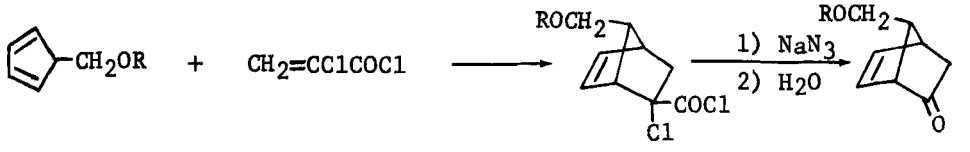
Carbocyclic and Heterocyclic Ring Formation - Halogen-substituted carbocyclic three-membered rings formed from olefins and phenyl(trihalomethyl)-mercury compounds are the subject of a recent review.[13] An improved organomercury route to *difluorocarbene* has been described.[14] *Cyclopropyl* compounds (or *aziridines*) can be prepared *via* the attack of a carbanion (or amine) on the readily obtainable dimethylaminophenyl-(2-phenylvinyl)-oxosulfonium fluoroborate followed by elimination of the sulfur grouping.[15]



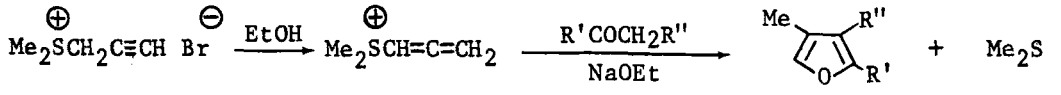
Diphenylsulfonium cyclopropylide[16] and the ylide derived from the N,N-dimethyl salt of cyclopropylphenylsulfoximine[17] reacts with α,β-unsaturated and β-dimethylamino ketones (in the latter case) to give high yields of *spiropentane* derivatives.



Certain nitriles, sulfones, and sulfonamides can be converted to their α,α-dilithio derivatives and then to *three-* and *five-membered ring compounds* with dihaloalkanes.[18] A 4+2 cyclo-addition of a $-CH_2CO-$ unit has been accomplished using 2-chloroacrylyl chloride.[19]



Furans are readily obtained according to the following scheme.[20]



A synthesis of β-*lactams* involves the addition of one mole of a primary amine to cyclopropanone (ketone + diazomethane) and treatment of the resulting carbinolamine with t-butyl hypochlorite followed by $AgNO_3$ in CH_3CN.[21] *3-Pyrrolidinones* have been prepared by the addition of azo-

methine oxides to allenes.[22] A one-step synthesis of *oxazoles* involves the reductive cyclocondensation of acyl halides and α-azidocarbonyl compounds in the presence of Ph_3P.[23]

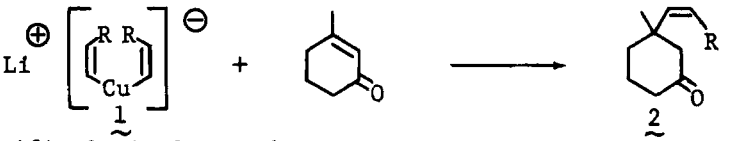
Reductions - For unsaturated compounds containing oxygen (allylic alcohols and ethers, ynols, epoxides, α,β-unsaturated ketones, aldehydes, acids and epoxides) *nickel boride* (prepared from Ni acetate and $NaBH_4$) selectively and quantitatively hydrogenates the C-C double bonds without rearrangements, hydrogenolysis or carbonyl reductions.[24]

An *aryl trifluoromethyl* group can be reduced to a methyl group with $LiAlH_4$ if an electron-donating amino or hydroxyl substituent is present in an *ortho* or *para* position.[25] Aromatic *diazonium fluoroborates* are reduced to the corresponding arenes in DMF at <80° when catalyzed by rhodium complexes, $RhCl(Co)(PPh_3)_3$ or $RhCl(PPh_3)_3$.[26] Electron attracting substituents appear to favor this reduction. Reductive *decyanation* of alkyl nitriles may be effected in 58-100% yields by the action of $Fe(C_5H_7O_2)_3$ and Na.[27]

The remarkable selectivity of *sodium cyanoborohydride* ($NaBH_3CN$) as a reducing agent in aqueous or aqueous MeOH systems has been elaborated.[28] Oximes are smoothly reduced to alkylhydroxylamines, and enamines are reduced to amines under acid catalysis. Reductive amination of an aldehyde or ketone with $BH_3CN^{\ominus}$ in the presence of NH_3, 1° amine or 2° amine at pH 7 leads to 1°, 2° or 3° amines, respectively. Reductive amination of substituted pyruvic acids leads to fair yields of the corresponding α-amino acids. ^{15}N-labeling can be accomplished by using $^{15}NH_3$. Also the hydrogens of $BH_3CN^{\ominus}$ can be readily exchanged for either ^2H or ^3H, thus permitting the synthesis of ^2H or ^3H-labeled alcohols, amines and amino acids. Tosyl hydrazones of aliphatic aldehydes and ketones are selectively reduced to the corresponding hydrocarbons with this reagent; hydrazones of aromatic ketones are not reduced.[29] Primary and 2° iodo and bromo groups as well as 1° tosylates can also be reductively removed in the presence of practically all other functional groups with sodium cyanoborohydride in hexamethylphosphoramide (HMPH).[30]

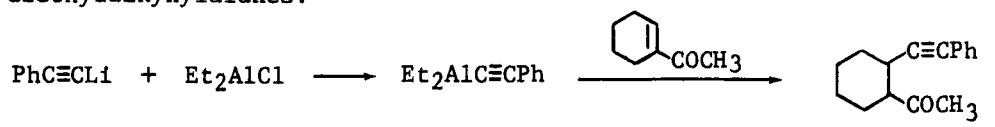
A single-step *reductive elimination* of epoxides to olefins with Zn-Cu couple in ethanol has been described. Sulfoxides, including the conformationally restricted thioxanthine sulfoxide, are quantitatively deoxygenated with $NaBH_4$-$CoCl_2$ in 95% ethanol.[31] Sulfoxides and sulfilimines are also reduced to sulfides at room temperature by O,O-diethyldithiophosphoric acid.[32]

Conjugate Addition Reactions - The conjugate additions of *lithium dialkylcopper* reagents (LiR_2Cu) have been further elaborated. In conjugated dienones, the presence of an alkyl group at one of the substitution sites directs the conjugate addition to the other position.[33] For 1-alkenylcuprolithium complexes, the *cis* or *trans* integrity of the alkenyl group is preserved during conjugate additions or in alkylations. Thus, 1 gives 100% 2.[34]

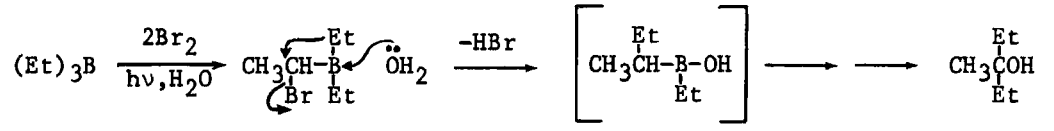


Stereospecific β-vinylation has been observed in a special case.[35]

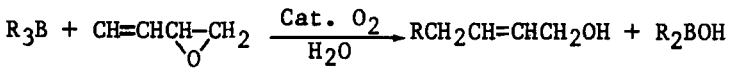
Acetylenic groups may be added to α,β-unsaturated ketones *via* diethylalkynylalanes.[36]



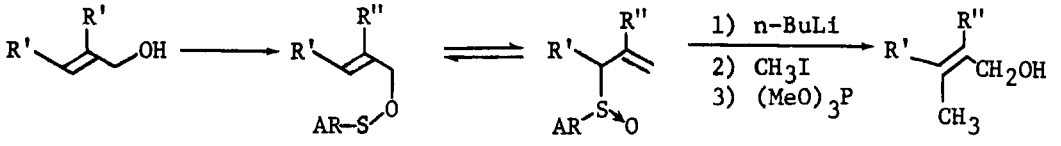
Alcohols and Alkyl Halides - Controlled oxidation of the hydroboration product of olefins of the type RR'C=CHR" with pure O_2 followed by hydrolysis of the trialkylborates gives the corresponding alcohols R(R')CHCH(R")OH.[37] Trialkylboranes are converted readily to *trialkylcarbinols* by reactions with chlorodifluoromethane under the influence of lithium triethylcarboxide.[38] *Tertiary* alcohols are also produced when trialkylboranes are photobrominated in the presence of water and the (rearranged) products subsequently oxidized.[39]



A one-step *four-carbon homologation* leading to a variety of 4-alkyl-2-buten-1-ols is provided by reaction of 1,3-butadiene monoxide with primary or secondary trialkylboranes in the presence of catalytic amounts of O_2 or other free-radical initiators.[40]

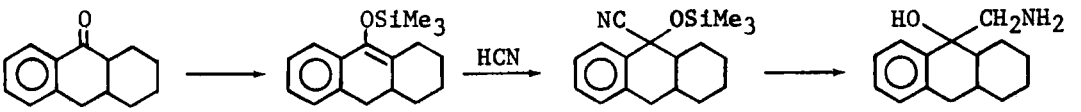


Chromic acid oxidation of bicyclo[3.3.1]nonane, bicyclo[3.2.2]-nonane, bicyclo[3.3.2]decane and adamantane affords the *bridgehead tertiary carbinols* which provide the best entry to functionalization at these sites.[41] Allylic alcohols can be transformed to their corresponding unrearranged *allylic chlorides* by the use of methanesulfonylchloride and a mixture of LiCl-DMF and s-collidine at 0°.[42] The reversible 1,3- transposition of sulfoxide and alcohol functions in an allylic system appears to be of general synthetic utility.[43]



Solvolysis of oxiranes with dimethyl sulfoxide and 2,4,6-trinitrobenzenesulfonic acid affords *threo-glycols* from *cis*-epoxides and

erythro-glycols from *trans*-epoxides.[44] A new route to β-*amino alcohols* from ketones is especially suitable for ketones which do not form cyanohydrins or condense with nitromethane.[45]



A new method for the conversion of alcohols to alkyl halides involves carbazate intermediates which are oxidized with N-halogenosuccinimides in the presence of pyridine.[46] *Gem-dichloroalkenes* have been prepared from the tosylates of aryl-, alkyl- and alkynyl(trichloromethyl)-carbinols by reaction with PhMgBr.[47] In another *anti-Markownikov hydrobromination* of internal olefins, the olefin is condensed with 9-borabicyclo[3.3.1]nonane and the intermediate subjected to α-bromination-HBr cleavage.[48]

Amines and Azides. A wide variety of organic azides has been converted to *alkylethylamines* by reaction with triethyl borane followed by methanolysis of the intermediate.[49] Enamines may be reduced to the corresponding

$$R\text{-}\overset{\ominus\oplus}{N}\text{-}N{\equiv}N + Et_3B \xrightarrow{-N_2} R\text{-}\overset{Et}{N}\text{-}BEt_2 \xrightarrow{MeOH} RNHEt + Et_2BOMe$$

tertiary amines with $NaBH_4$ *via* the C-mercurated immonium salts.[50]

A general method for exhaustively alkylating amines to *quarternary ammonium compounds* at room temperature makes use of the sterically hindered base 1,2,2,6,6-pentamethyl piperidine.[51] Cyclic or acylic higher olefins may be aminomethylated with CO, H_2O, and a secondary amine in the presence of rhodium oxide (and/or iron pentacarbonyl).[52]

Cycloaliphatic azides can be prepared at room temperature by the reaction of the Li amide of the primary cycloaliphatic amine with tosyl azide.[53] Phenyliodosochlorideazide [PhI(Cl)(N_3)] is a reagent which converts olefins to *chloro-azido* compounds, generally adding *cis* to double bonds, whereas ClN_3 adds *trans*.[54] α-*Azido*-β-*nitratoalkanes* have been produced when olefins were treated with sodium azide and ceric ammonium nitrate in acetonitrile.[55]

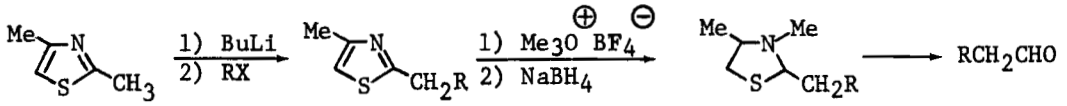
Aldehydes and Ketones - *Aldehydes* are obtained from terminal acetylenes according to the scheme:[56]

$$RC{\equiv}CH + R_3SiH \longrightarrow RCH{=}CHSiR_3 \longrightarrow RCH\text{-}CHSiR_3 \xrightarrow[CH_3OH]{H_2SO_4} RCH_2CHO$$
(O)

Several labile aldehydes have been prepared from alkyl halides and methylthiomethyl sulfoxide.[57]

$$CH_3SOCH_2SCH_3 + RX \xrightarrow{NaH} RCH(\overset{O}{\rightarrow}S\text{-}CH_3)(S\text{-}CH_3) \xrightarrow{\overset{\oplus}{H}} RCHO + CH_3SSCH_3$$

The conversion $RX \longrightarrow RCH_2CHO$ may be accomplished utilizing commercially available 2,4-dimethylthiazole.[58]

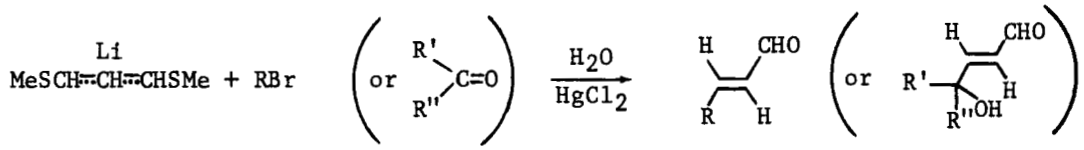


Ferrate(VI) ion (K_2FeO_4) rapidly oxidizes primary amines and alcohols to aldehydes and secondary alcohols to ketones without affecting double or triple bonds, aldehyde groups, or tertiary alcohol or amine functions.[59] Oximes are converted to aldehydes and ketones virtually instantaneously with $Tl(NO_3)_3$.[60]

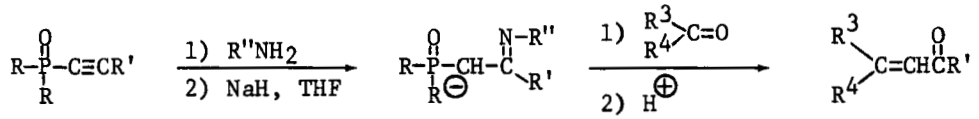
Ketones are conveniently obtained from olefins by oxymercuration, transmetallation with palladium chloride and subsequent decomposition of the organopalladium intermediate.[61] Ketones can also be prepared from the corresponding nitro compounds using aqueous $TiCl_3$.[62] Symmetrical alkyl or aryl ketones are formed from organomercuric halides and $Ni(CO)_4$.[63] *Highly-branched carbonyl compounds* are obtained *via* the 1,4-addition of B-alkylboracyclanes with bulky alkyl groups to α,β-unsaturated carbonyl compounds.[64]

The conversion of aldehydes to ketones can be accomplished by protecting the derived aldehyde cyanohydrin with ethyl vinyl ether and alkylating this species with lithium diisopropylamide and an alkyl halide following by deprotection.[65]

A procedure for the *vinylogous homologation* of aldehydes (RCHO $\longrightarrow$ RCH CHCHO) has been developed.[56] The reagent 1,3-*bis*(methylthio)-alkyllithium reacts with carbonyl compounds (or alkyl halides) to afford the corresponding unsaturated *trans* aldehydes.[66] The α-hydrogen of an



α,β-unsaturated ketone can be replaced by an alkyl group *via* a metalloenamine.[67] *Allylic oxidation* of trisubstituted olefins by SeO_2 has been established to give selectively *trans* allylic alcohols, or *trans* α,β-unsaturated aldehydes in certain cases.[68,69] A new synthesis of α,β-*unsaturated ketones* involves a Wittig type addition to a ketone and hydrolysis of the resulting ketimine.[70]



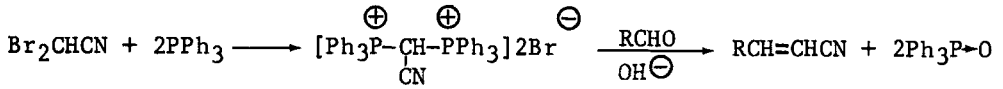
Disubstituted alkynes are oxidized to α-*diketones* by NaOCl (or $NaIO_4$) with a catalytic amount of RuO_4.[71] Symmetrically disubstituted acyclic olefins or large ring alicyclic olefins are directly converted to α-diketones by $KMnO_4$-Ac_2O in the cold.

Carboxylic Acid Derivatives - Stable lithium ester enolates, prepared by reaction of carboxylic esters with lithium N-isopropylcyclohexylamide (LiICA) in THF at low temperatures, may be potentially more versatile than sodiomalonic ester in synthetic applications. The coupling reactions are carried out at room temperatures or below and give good yields of the desired products. Thus, the lithium ester enolates may be a) alkylated to the corresponding *alkylated ester*,[73] b) carboxylated with CO_2 to produce *malonic ester* derivatives,[74] c) halogenated to α-*iodo* and α-*bromo esters*,[75] and d) acylated with acyl chlorides to give the corresponding β-*keto esters*.[76] The lithium salts of the α-lithiated carboxylic acids have been condensed with esters to give β-*ketoacids*, and added in Michael fashion to α,β-unsaturated esters.[77] β-*Hydroxy-acids* are produced directly when these α-lithiated carboxylic acid salts are condensed with ketones.[78]

The *chain-lengthening* of carboxylic acids by three carbon atoms to γ-*keto acids* (or nitriles) by way of oxazoline-5-one anions have been described.[79] The conversion of aldehydic cyanohydrins to α-hydroxy-N-t-butylcarboxamides followed by oxidation and hydrolysis constitutes a new general procedure for the preparation of α-*ketoacids*.[80]

A mild general procedure for preparing *peroxy acids* in 70-85% yields involves the acid catalyzed perhydrolysis of acyldiethylphosphates.[81]

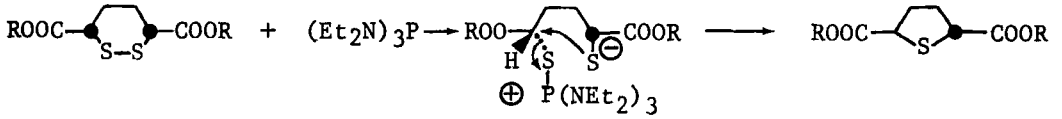
Nitriles have been prepared from a) carboxylic acids in one step by heating with amidosulfuric acid and urea,[82] b) aldoximes by reaction with $TiCl_4$ in the presence of pyridine,[83] and c) aldehydes *via* a modified Wittig reaction with cyanomethylene-*bis*(triphenylphosphonium) dibromide, this reaction giving rise to α,β-*unsaturated nitriles*.[84] α,β-Unsaturated



aldehydes have been converted oxidatively to *amides* without affecting the olefinic bond by treatment with manganese dioxide in the presence of NaCN and an amine.[85]

A new *isocyanide* synthesis involves simply heating a monosubstituted aliphatic or aromatic formamide (readily prepared at 0° from the amine and phenylformate[86]) in $CHCl_3$ or CH_2Cl_2 with triphenylphosphine, CCl_4 and Et_3N.[87]

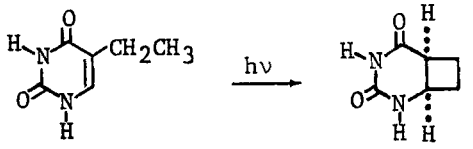
Miscellaneous - Dialkyl, aralkyl, alicyclic and certain diaryldisulfides undergo facile *stereospecific desulfurization* with *tris*(diethylamino)-phosphine. Inversion of configuration occurs at one of the carbon atoms α to the disulfide group.[88] Some biologically important disulfides such



as derivatives of lipoic acid and cystine have been desulfurized in high yield to the corresponding thioethers.[89]

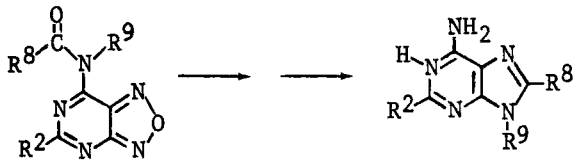
A two-step process for preparing *N-monoalkylsulfonamides* from the alkyl bromide without going through the alkylamine involves the alkylation of the sodio derivative of the ethyl N-(arylsulfonyl)carbamate and subsequent hydrolysis of the carbamate.[90]

The *Claisen rearrangement* has been applied to prepare 6-allyl-5-hydroxyuridine from 5-allyloxyuridine in 79% yield.[91] The reaction is general for the 5-allyl ethers of hydroxyuracils and methods are available for the eventual removal of the 5-hydroxy group. A *new class of dihydropyrimidines, viz.*, analogs of 5,6-dihydro-5,6-cyclobutanyluracils and their nucleosides, are obtained when aqueous solutions of 5-alkyl uracils (ethyl, propyl, isopropyl) or their nucleosides are irradiated at 254 nm.[92]



The conversion of uridine or its analogs or derivatives to cytidines has been accomplished in one step by heating the uridine in an autoclave with hexamethyldisilazane and ammonia.[93] The 5'-hydroxy group in purine and pyrimidine ribonucleosides has been selectively replaced with Cl or Br at room temperature with $SOCl_2$ (or $SOBr_2$) in HMPA.[94]

A novel synthesis of *2-, 8- and 9-substituted adenines* where the substituents are introduced unambiguously has been described.[95] The reactions start with 4,6-diamino-5-nitrosopyrimidines either substituted or unsubstituted in the 2-position and the eventual adenine 8- and 9-substituents are introduced on the intermediate 7-aminofurazano[3,4-d]-pyrimidine. A new approach to the synthesis of *8-azapurine nucleosides* involves opening the imidazole ring of the appropriately protected pre-formed purine nucleosides and cyclization of the resultant 5-amino-4-glycosylaminopyrimidine with nitrous acid.[96]



The difficultly accessible *3-substituted pyridines* have been synthesized in one step by alkylation (or bromination) of lithium tetrakis(N-dihydropyridyl)aluminate, which is readily prepared by the reduction of pyridine with $LiAlH_4$.[97]

The diazonium fluoroborates prepared *in situ* from 2- and 4-aminoimidazoles have been photolyzed at room temperature or below to give *2- and 4-fluoroimidazoles* in fair yields. This reaction, which did not take place in the absence of u.v. light even under pyrolytic conditions, has been applied to the synthesis of 4-fluoro-DL-histidine.[98]

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Chapter 26. Intramolecular Catalysis in Medicinal Chemistry

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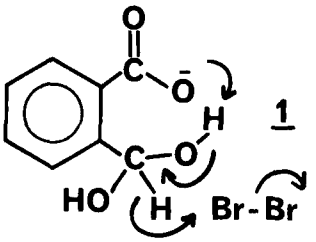
Intramolecular catalysis is important in medicinal chemistry because (1) it is a feature of the reactivity of multifunctional molecules, (2) it plays a special role in the design and behavior of latentiated drugs, and (3) it serves as a model for catalysis by active-site functionalities in reactions of enzyme-substrate complexes. During 1971, excellent reviews of the subject appeared by Bender,[1] Capon[2] and Fersht and Kirby,[3] which dealt critically and comprehensively with previous work. This article concentrates on developments during 1971 and is highly selective. It is organized according to the type of reaction being catalyzed, in order of increasing complexity: proton transfer from carbon; acetal hydrolysis; acyl transfer; hydrolysis of phosphate esters. At the end, papers contributing to the "orbital steering" controversy are reviewed. Throughout, the abbreviation "TS" is employed for "transition state."

Hydrogen Transfer From Carbon- Deuteron transfer to the intramolecular $(CH_3)_2N$ group in $(CD_3)_2C{=}NH^+CHR_1CHR_2CH_2N(CH_3)_2$ leads to 7x to 100x rate enhancements in the dedeuteration of $(CD_3)_2CO$, catalyzed *via* the immonium ions by the corresponding diamines. Only amines capable of forming an 8-ring cyclic TS are effective and only then in cases where the catalytic function is *syn* about the immonium center to the proton donor group.[4] The 8-ring TS is not general for intramolecular proton transfer from carbon. For example, a 6-ring TS is favored for proton transfer to the carboxylate center in $CH_3CHNO_2CH_2CH_2CO_2^-$ to form the nitrocarbanion.[5] Hydride transfer is intramolecularly base-catalyzed in the bromine oxidation of *o*-formyl benzoate ion to produce a 70x acceleration (other substituents affect the benzaldehyde rate only within 3x). Nucleophilic addition of $-CO_2^-$ to the formyl group is excluded because phthalic anhydride hydrolyzes too slowly to be an intermediate; presumably the hydrate intervenes (1). The substrate exists as the lactol in acidic solution and then oxidizes at least 10x more *slowly* than benzaldehyde.[6] Intramolecular acid catalysis of hydride transfer by Zn^{++}-activation of the hydride acceptor gives a rate constant of $19 \pm 4\ M^{-1}sec^{-1}$ to the alcohol-dehydrogenase model reaction 2, while no reaction at all is observed in the absence of Zn^{++}. This is the first non-enzymatic reduction of an aldehyde function by a dihydropyridine.[7]



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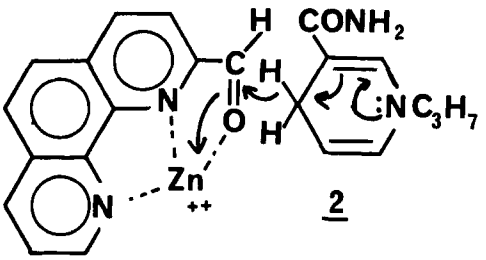

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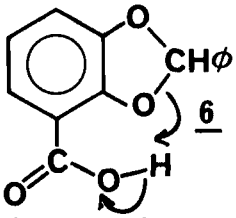
Acetal Hydrolysis- Acid-base catalysis of acetal hydrolysis is important for the mechanism of glycosidase action.[2,8]

Carboxyl groups might stabilize the hydrolysis TS by (a) partial proton donation (general acid catalysis); (b) complete, prior proton donation to the acetal linkage, followed by electrostatic interaction of $-CO_2^-$ and the positive acetal linkage during CO bond fission (electrostatic catalysis), or (c) prior proton donation followed by covalent binding of $-CO_2^-$ to the incipient carbonium center during CO fission (nucleophilic catalysis). Recent work bears on all of these.

Acetals were thought subject only to specific acid catalysis until it was shown that if the RO- group to be protonated were unusually weakly basic or if the carbonium ion left by RO- departure were unusually stable then early cleavage and general catalysis would be seen. Intramolecular carboxyl catalysis is promoted by the same features which favor the intermolecular reaction; current work has centered on whether the mechanism is general acid, electrostatic or nucleophilic catalysis. The charge δ+ in the incipient carbonium ion of TS 3H (i.e., 3 with Y = H) is the same ($\rho^* = -3$ for variation at R) whether X = CH_3, H or -. From this it was concluded that there was no direct TS interaction of the CO_2X group and *either* the OH group or the incipient carbonium ion (thus no general catalysis and no nucleophilic catalysis, leaving electrostatic catalysis as the only explanation of the 500x acceleration when X = -.[9]) The conclusion is valid if hydrogen-bonding of CO_2^- to the OH would alter δ+, as seems reasonable. On the other hand, the electrostatic hypothesis to a first approximation would not place any orientation requirement on the carboxylate group; but 2-methoxymethoxy-1-naphthoic acid (in which coplanarity of the $-CO_2H$ is prevented) is hydrolyzed 5x more slowly than the reaction of 3H, while steric pressure on the acetal group which does not interfere with the carboxyl orientation (as in $3CH_3$, $3NO_2$, and $3CO_2H$, as well as 1-methoxymethoxy-2-naphthoic acid) gives accelerations of 4x to 490x over 3H.[10] Furthermore, compound 4 is estimated, assuming no stabilization by the CO_2^- negative charge, to be too unstable an acid to be an intermediate in the fast observed reaction. Only if its K_a is decreased at least 1000x by the $-CO_2^-$ is the electrostatic-catalysis mechanism possible. If the stabilization involves hydrogen bonding, the mechanism is in effect general catalysis, as favored by the authors.[11] Electrostatic catalysis is invoked to explain the 30x acceleration in 5 over the glucosides, in which no $-CO_2^-$ group is present. Here the carboxylate is stereochemically ill-situated for either general or nucleophilic catalysis.[12] Just as the benzylidene catechols experience intermolecular catalysis,[13] so their hydrolysis is also accelerated intramolecularly (6) either by electrostatic or general catalysis, since the carboxyl group is in no position to effect nucleophilic stabilization at the incipient positive carbon.[14] Benzaldehyde disalicyl acetal (7) hydrolyzes more than 10^9x faster than its dimethyl ester, while the monoanion forms the acylal another 65x faster.[15] While the cyclization does not prove TS-participation by $-CO_2^-$, it is



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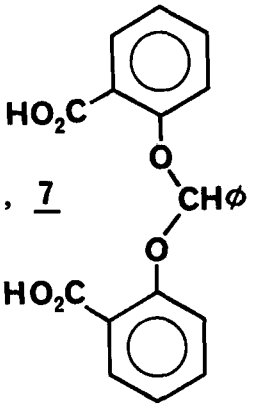
suggestive. A search for nucleophilic assistance by carboxylate and other centers yielded cyclized products in some cases, but generally insufficient rate accelerations to justify postulation of TS carboxylate assistance except in 50-82% dioxane-water solutions. For example, 2-carboxybenzaldehyde diethyl acetal hydrolyzes 3000x faster than the 4-carboxy isomer in basic 82% dioxane-water but only 3x faster in water. The origin of the dioxane effect is uncertain.[16]



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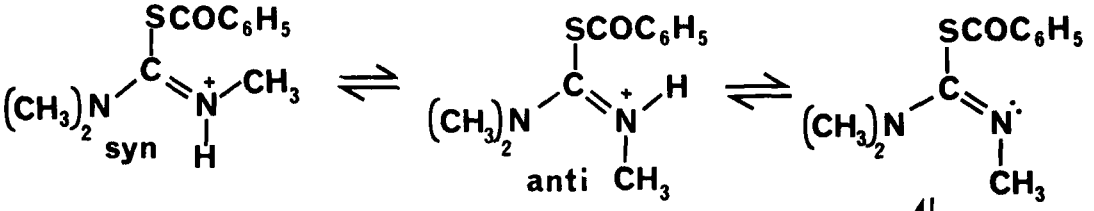
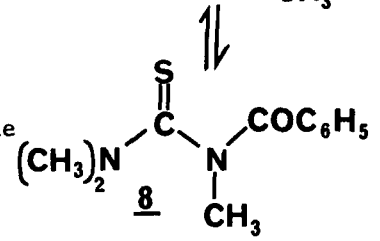
Acyl Transfer- Intramolecular catalysis of these processes has been a subject of interest for years, partly because of interest in acyl-transfer enzymes. Among the new examples are the 10x acceleration produced by the *o*-O^- group in the basic hydrolysis of *o*-hydroxyphenyl ethyl carbonate (over the *o*-OCH_3 and *p*-O^- compounds)[17] and >150x enhancement by $-CO_2^-$ in hydrolysis of $C_6H_5O_2CCH_2-CO_2^-$.[18] Both reactions are thought to proceed by general base catalysis.

Intramolecular acyl shifts (which are important *per se*, and which also occur as steps in nucleophilic catalysis) include the biotin model reaction 8 simulating the presumed 1,3-O,N shift of CO_2 in biotin.[19] At



pH < 9, where the substrate is protonated, the *syn-anti* isomerization is fast and acyl transfer in the free base is rate determining (overall base catalysis). Above pH 10 the free base is the major species and is frozen in the unreactive *syn* form; it reprotonates, isomerizes to *anti* in a rate-determining step and the shift follows rapidly (overall acid catalysis). Thus there is a rate maximum at pH 9-10.[20] The closure of *o*-hydroxymethylbenzamide to phthalide is catalyzed by imidazole, yielding a close model for acylation of chymotrypsin.[21] An interesting competition of general-base and nucleophilic catalysis is found for 9. The hydrolysis rate increases with the degree of ionization of the carboxyl group below pH 8, with formation of succinic anhydride (nucleophilic catalysis by $-CO_2^-$). Above pH 8, there is a second sigmoid increase, paralleling that for catechol monoacetate (in both cases $^kH_2O/^kD_2O$ = 1.8) and owing to general-base catalysis by $-O^-$.[22,23]



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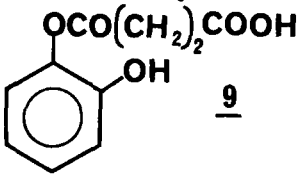

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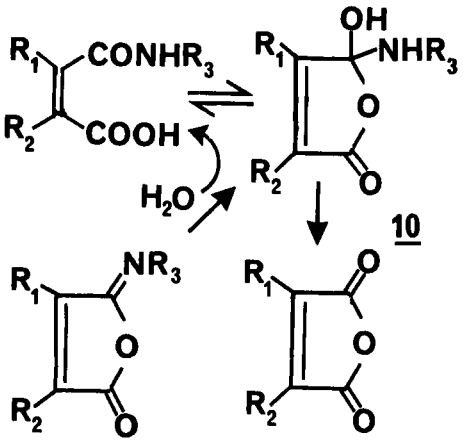
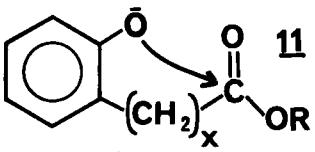
Acyl Transfer: Alkyl Effect- The kinetic and thermodynamic driving of ring closure by alkyl substituents

in the succinyl moiety of compounds like 9 is well-known. Log k for the nucleophilic catalysis of hydrolysis of 9 parallels values of log K_{eq} for anhydride formation from alkylated succinic acids with a slope of 0.53 (TS receiving 53% of the equilibrium driving force).[23] With succinanilides (intramolecular general *acid* catalysis), where expulsion of the leaving group should be more difficult, the corresponding slope is 0.75, consistent with the expected more product-like TS.[24] Previous work has tended to emphasize conformational factors as the origin of the alkyl effect in these systems, but semiquantitative estimates of the energetics of nonbonded interactions suggest relief of initial-state strain as the main contributor.[24] Compelling evidence for this view appears in the enormous accelerations experienced by dialkyl substitution in maleamides (10) where no question of conformational isomerism arises.[25] Intramolecular general-acid catalysis of anhydride formation proceeds with rate determining decomposition of the cyclic adduct (shown by alternative generation of the adduct from the isoimide, which gives only the maleamic acid--thus the adduct returns to reactant faster than going on to product). Monoalkylation of the double bond produces moderate, apparently electronic effects. Dimethylation ($R_1 = R_2 = CH_3$), however, produces a 23,500x acceleration! The effect appears as a 10 kcal/mol reduction in ΔH^*, opposed by a 10 eu decrease in ΔS^*. Inclusion of the two alkyl groups in a 5- or 4-ring completely reverses the effect: the half amides of cyclopentene and cyclobutene dicarboxylic acids react, respectively, 10^4x and 10^5x more *slowly* than the parent compound. Since all the anhydrides hydrolyze at very similar rates, the effect is presumed to originate in initial-state strain relieved in the transition state and product.[25] Interestingly, K_{eq} for dimethylmaleic anhydride formation from the acid appears to be only about 530x larger than K_{eq} for maleic anhydride formation,[24] a factor of 45x smaller than the acceleration of amide closure. Perhaps an electronic requirement for near coplanarity of the amide function and the double bond intensifies the initial state strain, or perhaps a near-tetrahedral center in the TS leads to greater strain relief there than in the anhydride. The generally small effects of monosubstitution are confirmed by the narrow range of rates of ring closure of various α- and β- substituted coumaric acids.[26]



Acyl Transfer: Other Structural Factors- Ring-size preferences in the TS are expected (and are found) to vary with the catalytic mechanism. The aminolysis of acetylimidazole by diamines shows 100x rate enhancements with 2 or 3 methylene groups between the nucleophilic and presumed base-catalytic nitrogens, while 4- or 5- atom bridges gave only 15x accelerations.[27] In the very large effects produced by internal nucleophilic attack in 11 (more than 10^5x faster than the corresponding intermolecular reaction), the 5-ring TS (x = 1) is preferred by a small factor, 5x in rate, over the



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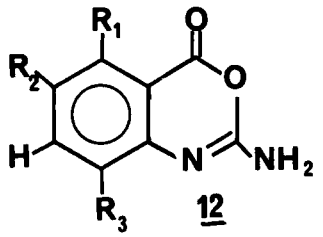
6-ring TS (x = 2).[28] The same is true for general-base catalyzed lactonization of ω-hydroxyalkanoic aryl esters, in which the butyrates cyclize 10x - 20x faster than the valerates. By contrast, the valerates undergo acid-catalyzed hydrolysis probably *via* the lactone about 2x as fast as the butyrates.[28] The hydroxyl group appears not to participate in the acidic hydrolysis of the conjugate acid of 11. The placement of potentially catalytic groups is a matter of obvious importance. Thus location of a carboxyl group at R_1 in 12 gives hydrolysis rates (water attack at carbonyl) little different from those for carboxyl at R_2, while carboxyl at R_3 gives an enhancement of at least 10x. Apparently protonation of the leaving-group nitrogen is more important than proton donation to the carbonyl oxygen.[29]



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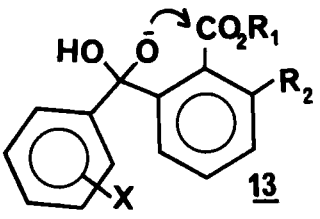
Catalytic power may be conferred on otherwise inert functional groups by an activating chemical reaction. For example, hydroxide ion adds to the keto function of *o*-acyl esters to produce an intramolecular basic group (13) which in subsequent rapid steps produces hydrolysis of the ester.[30] *Cis*-3-benzoylacrylates enjoy a similar catalysis of hydrolysis with benzoyl substituents giving ρ ∿ 2-2.5, while in *trans*-3-benzoylacrylates, no such possibility exists and ρ ∿ 0.6-0.7.[31] Another example of activation is provided by esters of 3-hydroxybutyric and *cis*-2-hydroxycyclopentanecarboxylic acids, in which esterification of the hydroxyl groups by borate leads to intramolecular borate catalysis of hydrolysis of the ester function. The hydroxyl groups by themselves produce only a small acceleration, perhaps connected with solvent structuring.[32]



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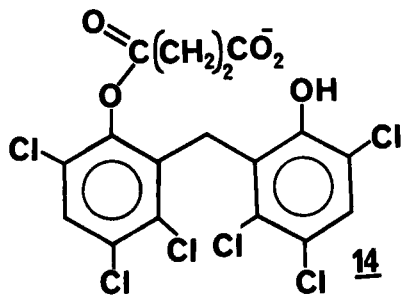
Acyl Transfer: Bifunctional Catalysis- Many mechanisms for enzyme catalysis postulate cooperative ("push-pull") catalysis by an acid-base pair in the active site. The analogous bifunctional intramolecular catalysis has been widely sought; the evidence presented generally consists of a maximum rate at a pH sufficiently acidic for the acid-catalytic function to be protonated yet sufficiently basic for the base-catalytic function to be free. The hydrolysis of hexachlorophene monosuccinate (14), for example, exhibits such a rate maximum at pH 6.8 (between pK_a 5.20 for the carboxyl group and 8.4 for the phenolic group). Succinic anhydride can be trapped, demonstrating a nucleophilic role for $-CO_2^-$. The -OH is presumed a general acid. Hexachlorophene monoacetate hydrolyzes 500x faster than the diacetate giving a rough measure of the -OH catalysis alone, while the monoanion of 14 reacts 3 x 10^4x faster than the monoacetate, a measure of the nucleophilic-plus-cooperative contribution.[33] This system contrasts with the



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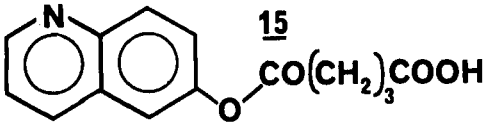
competing nucleophilic and general base catalysis by $-CO_2^-$ and $-O^-$ seen for 9 above.[22] A rate maximum at pH 2.5 for the hydrolysis of methyl 2,6-dicarboxybenzoate was taken to indicate bifunctional catalysis. The monoanion, however, reacted only 12x faster than the neutral ester which was explained by the greater importance of leaving-group protonation than of base catalysis in the probably rate-determining breakdown of the tetrahedral intermediate.[34] A very severe blow was dealt the use of bell-shaped pH-rate profiles as evidence for bifunctional catalysis by the observation of such curves in the hydrolysis of molecules like 15 and p-$HO_2CC_6H_4OCO(CH_2)_2CO_2H$, where no direct participation by the second function is stereochemically possible.[35] In 15, protonation of N increases the rate by improving the leaving group; deprotonation thus reduces the rate ("electronic inhibition"). Similarly deprotonation of the p-CO_2H group in p-carboxyphenyl succinate produces "electronic inhibition." Even when the group is stereochemically able to catalyze, its effect may be electronic.[35] This criticism applies to the two examples cited above. In fact, the postulated acidic bifunctional component in several cases of this kind was shown to obey a linear free energy relation defined by remote substituents, suggesting a non-catalytic role.[35]



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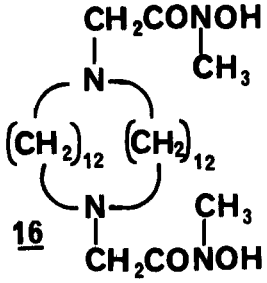
Acyl Transfer: Complexation- Intramolecular catalysis within a reversibly-formed complex offers a strong analogy to reactions within the enzyme-substrate complex. Complexing agents such as caffeine, various alkyl ammonium ions, piperazinedione and triglycine exhibited reversible binding (water, pH 4.6) with the O-acetyl, hexanoyl and octanoyl esters of 8-hydroxyquinoline but the attack of water on the ester carbonyl, general-base catalyzed by the quinoline nitrogen, was *reduced* in rate in the complex. In contrast, direct complexation (inferred from chain-length effects; no saturation kinetics observed) between the quinoline esters and long-chain alkyl amines increased the rate of the correspondingly catalyzed aminolysis reactions. Decylamine reacts 20x-40x faster than ethylamine with the quinoline esters, and reacts more than 2x faster with the 8-derivative (capable of intramolecular catalysis) than with the 6-derivative.[36] The macrocyclic hydroxamic acid 16 contains a hydrophobic binding site (the cavity of the ring) and two functions for intracomplex reaction. Saturation kinetics is observed with p-nitrophenylbutyrate (K_m = 1 *mM*) even though the medium contains 11% methanol and 2% acetonitrile. "Burst" kinetics is seen with all p-nitrophenyl esters, the size of the burst indicating that only one hydroxamic-acid group is active. The acceleration over the reaction rate with N,N-diisobutylglycine N-methylhydroxamic acid, produced by 16, rises from 1.7x (p-nitrophenyl acetate) to 7600x (p-nitrophenyl dodecanoate).[37]



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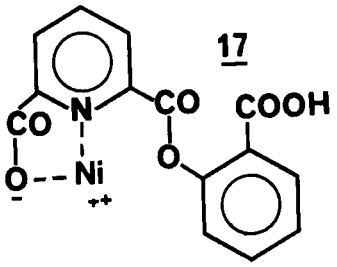
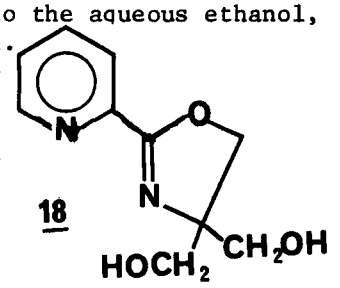
Complexation with Ni^{++} to form 17 gives a 56x increase in the ester-hydrolysis rate, while ionization of the salicyl carboxyl produces a further 1.7x increase, leading to the postulate of bifunctional (carboxylate-metal ion) catalysis. The carboxylate effect is in the wrong direction for



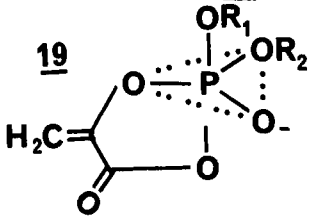
17

an electronic, non-catalytic explanation. The small bifunctional acceleration is termed "semi-cooperative."[38] The reaction of 2-cyanopyridine with water to give pyridine-2-carboxamide, catalyzed by Cu^{++} or Ni^{++}, is completely circumvented by addition of Tris buffer to the aqueous ethanol, 18 becoming the sole product. 18 results from an intracomplex reaction of the Tris-alkoxide conjugate base with 2-cyanopyridine while both are ligands on the metal ion. The metal is believed to acid-catalyze attack of the alkoxide oxygen on the nitrile group. Phenomenologically, the metal-ion catalysis appears in ΔS^{*}.[39]



18

Hydrolysis of Phosphate Esters- Intramolecular catalysis in the hydrolysis of second-row derivatives has figured large in the design of kinase and phosphatase mechanisms. The dominant theme has been the stereochemical limitations imposed by the intermediate trigonal-bipyramidal adduct,[40] exemplified by 19 for phosphoenol pyruvate. Electron-rich groups strongly prefer the equatorial positions of such species while only relatively electron-poor groups may attain the axial positions ("polarity rule"). Entrance to and departure from phosphorus must occur in axial positions. When R_2 = -(intermediate derived from $CH_2{=}C(CO_2^-)OPO_3R_1^-$), 19 is the only structure which can form because the two electron-rich $-O^-$ groups are locked equatorial and the 5-ring cannot span the equatorial positions ("strain rule"). Thus 19 can only return to reactants or expel R_1O^-. When R_1 = $C_6H_5CH_2$, expulsion of the latter (with acid catalysis) is the major (85%) reaction in spite of the expected greater leaving-group reactivity of the enol *vs* benzyl alcohol.[41] Protonation of one of the $-O^-$ also occurs (13%) to give OH which can occupy an axial position. Then isomerization of 19 to give 20 (pseudorotation[40]) is possible. Departure of the enol O in 20 (R_1 = H, R_2 = $CH_2C_6H_5$) gives an open-chain acyl phosphate which hydrolyzes to the observed pyruvic acid and monobenzyl phosphate. The dibenzyl substrate (yielding 19 or 20, R_1 = R_2 = $CH_2C_6H_5$) experiences no hindrance to pseudorotation and forms the open-chain acyl phosphate from 20 350x faster than it hydrolyzes. However, the open-chain compound nearly always re-closes and the chief (85%) overall reaction is again benzyl-alcohol expulsion. Kinetically, the hydrolysis reactions appear as pH-independent reactions of the phosphoenol pyruvic *acid* derivative (intramolecular general acid catalysis). Similarly, phenyl salicyl phosphate hydrolysis gives only phenol and salicyl phosphate (*via* 21, R_1 = C_6H_5, R_2 = -) while the triester formed from salicylic acid and propane-1,3-diol yields 21 with $R_1 + R_2 = (CH_2)_3$, which can undergo pseudorotation easily. The product above pH 5.3 is thus salicylic acid and propanediol phosphate; the reaction shows kinetic intramolecular general base catalysis. The rate determining step is probably the hydrolysis



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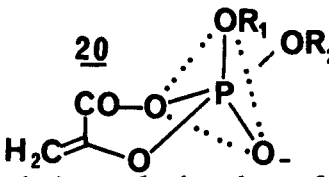

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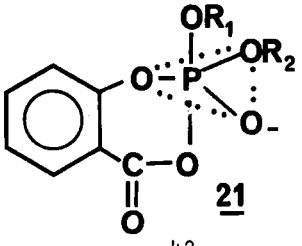
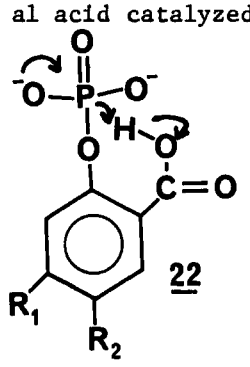


21

of the reversibly formed open-chain acyl phosphate derivative. Below pH 2.0, the predominant product is that from hydrolysis of one of the propanediol P-O bonds and kinetic intramolecular general acid catalysis is seen. The rate-determining step may now be acid-catalyzed cleavage of R_1O-P in 21, $R_1 + R_2 = (CH_2)_3$. The accelerations by both $-CO_2^-$ and $-CO_2H$ are 10^7x, relative to phenyl dialkyl phosphates.[42]

A long-standing point of interest has been the intramolecular general acid catalyzed expulsion of PO_3^- from salicyl phosphate (22), which shows no deuterium solvent isotope effect in spite of the apparent TS hydrogen bonding or proton transfer.[43] SO_3 expulsion from salicyl sulfate in a presumably similar process also shows no solvent isotope effect.[44] This lack has been ascribed in both cases to a product-like structure for the proton-transfer process in the TS (complete transfer to O leaving $-CO_2^-$). A crucial experiment has now disproved this hypothesis. Substituents at R_1 and R_2 in 22 bear a particular relationship to the phenolic and carboxyl functions: R_1 is *para* to $-CO_2H$ and *meta* to $-OPO_3^=$ and R_2 is *vice versa*. The rates of hydrolysis of substituted compounds may therefore be fitted to a two-term Hammett equation $\log k/k_o = \rho_1\sigma_1 + \rho_2\sigma_2$ where ρ_1 represents the substituent effect at the carboxyl and ρ_2 the effect at the phenolic center, with σ_1 and σ_2 appropriately chosen. The values are $\rho_1 = -1$ and $\rho_2 = +1.74$. The former shows the $-CO_2H$ to be intact as such in the TS (the substrate is in the form $R_1R_2C_6H_2(CO_2^-)OPO_3H^-$). Thus the lack of isotope effect is from a *reactant*-like TS for proton transfer; this is confirmed by ρ_2, which indicates a large negative charge at the TS phenolic center. The origin of the catalytic effect is suggested to be rotation of the carboxyl into the ring plane simultaneous with P-O fission, and resulting delocalization of the phenolic charge.[45]



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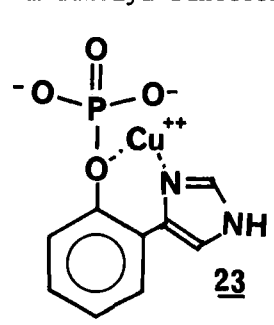
Metal ions are generally required for enzymic phosphoryl transfer. Their catalytic effects on phosphate ester and amide[46] hydrolysis in model systems are, however, quite modest in the absence of an intramolecular binding site. Given the latter, large accelerations are seen. An *o*-imidazolyl function binds Cu^{++} as in 23 to yield a greater than 10^4x acceleration of PO_3^- expulsion over the rate of the reaction without Cu^{++}.[47] The analogy to 22 above is interesting. Phosphoryl donation from an imidazolium to an oximate center is accelerated about 10^3x (*vs* hydrolysis of phosphorylimidazole rather than oximate attack) by the agency of Zn^{++} (24). The reaction is considered a good model for nucleoside diphosphokinase, which requires Mg^{++} and passes through a phosphorylhistidine intermediate.[48]



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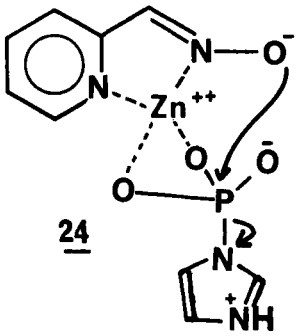
Orbital Steering- The idea that enzymes might derive



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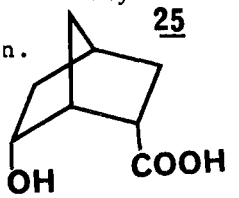
very large accelerations from effecting the precise orientation of reacting groups ("orbital steering") arose from the observation that the acid-catalyzed lactonization of 25 is 10^6x faster than ethanol-acetic acid esterification. An attempted dissection of this and related accelerations into contributions from approximation, strain and restriction of rotations left a factor of 2 x 10^4 unaccounted for which was assigned to orbital steering.[49] However, different corrections for strain and rotational restriction suggest that lactonization of 25, rather than being accelerated 45x relative to γ-hydroxybutyric-acid lactonization by orbital steering, is *retarded* about 15x.[50] Still another set of corrections produces a similar conclusion.[51] Both statistical-mechanical calculations[52] and rough entropy estimates[50] lead to accelerations of up to 10^6x-10^9x, merely for intramolecular *vs* intermolecular reaction, while only 55x was originally used. The general question of proximity effects was critically reviewed last year.[53]



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If reactions are greatly speeded or slowed by small changes in the orientation of groups in the transition state, the "ideal" transition state must be very rigid, at least near the reacting center. Force-constant estimates based on an orbital-steering factor of 10^8 show the requisite force constants to be far too large, about 100x larger than those in stable molecules.[51] A collision-theory calculation appears, however, to allow accelerations of about 30x-100x per reacting group,[54] for "precise" orientation within 10° of solid angle.[55]

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Chapter 27. Peptide Synthesis

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This review will summarize progress in methods of peptide synthesis and the synthesis of certain specific peptides and proteins from the literature since these topics were last reviewed in the Annual Reports for 1969. Two useful books[1,2], five symposium reports[3-7], and five useful reviews[8-12] have appeared.

Synthetic Methods - Preparation and use of enamine protecting groups[13] and a new protecting group based on o-nitrophenol[14] have been described. Procedures for removal of Boc groups with mercaptoethanesulfonic acid[15], which should be useful for protection of tryptophan, as well as for removal of trifluoroacetyl (TFA) and trichloroacetyl groups by reduction with $NaBH_4$[16] have appeared. The imidazole of histidine appears to be most effectively blocked by the tosyl (Tos) group,[4,17] although the 2,4-dinitrophenyl (Dnp) group is good[18]. Both of these groups inhibit racemization of histidine[7]. The Tos group is removed by HF, while the Dnp is not. The Tos group for the guanidine of arginine appears to offer advantages over the nitro group, since the former does not cause contamination of products with ornithine, due to decomposition of the arginine[19]. The guanidino-tosyl group is also cleaved by HF. A new group, diisopropylmethoxycarbonyl, ("Dipmoc") has been proposed for the ε-amino group of lysine, since it is more stable than the carbobenzoxy group and should avoid the problem of branching at lysine in solid phase synthesis[20]. However, this group appears to be more stable than would be ideal. Simultaneous deblocking and oxidation of cysteine in peptides can be accomplished with I_2 if the acetamidomethyl group is used for protection[21]. The S-isobutoxymethyl protecting group for cysteine[22] has also been described. Papers have appeared on the use of 2-pyridyl esters[23] and 2-pyridyl thioesters[24] in synthesis.

Simultaneous deblocking of o-nitrophenylsulfenyl peptides and coupling are possible using the method of Mukaiyama[25], which has now been shown to be racemization-free. The Merck method of synthesis with N-carboxy anhydrides in aqueous solution[26,27] has been described in detail. Coupling of peptides with DCC in the presence of hydroxybenzotriazole[28] has been shown to reduce racemization significantly. Cleavage of peptides at Thr-Pro bonds[29] in $Na-NH_3$ rules out this reagent as a general one for peptide synthesis, although some peptides are remarkably resistant to damage. A careful study of a facile N-O shift in insulin has appeared[30].

Racemization in peptide synthesis has been reviewed[9], and Manning has improved his method for detecting racemization by a correction for racemization during hydrolysis[31].

Solid Phase Synthesis (SPPS) - In the eight years since the introduction of this new technique, achievements in synthesis of peptides and even proteins by this method have been impressive. However, much uncritical work and naiveté of approach on the part of many investigators has seriously hampered development of the method.

Although many peptides have been synthesized successfully on the conventional polystyrene resin, particularly when the 1% crosslinked resin is used, it is apparent that new types of solid supports are needed, especially for the synthesis of large peptides and proteins. Recent work has reported the use of macroreticular resins[32,33], resin coated glass beads[34], polystyrene grafted onto a halocarbon nucleus[35], and even peptides bound covalently to glass beads[36]. New types of linkage to a conventional polystyrene resin involve the use of haloacyl resins, from which the peptide can be removed readily by nucleophilic reagents[37], and the use of peptide-resin bonds which are very stable during the synthesis, but can be readily modified to derivatives which are very labile[38-40]. Unfortunately, none of the systems proposed so far appear to be universally applicable. Attachment of the peptide to the resin via the ε-amino group of lysine was used in a novel synthesis of vasopressin[40a]. Loffet has proposed the use of a quaternary base in the usual esterification procedure for attachment of the first amino acid to the resin[41]. This should eliminate formation of quaternary ammonium groups on the resin, which may complicate synthesis as well as monitoring during the synthesis.

Incomplete removal of Boc groups during SPPS has long been recognized as a source of difficulty[42]. Unfortunately the solution found by Merrifield at that time, the use of 1% crosslinked resin and a deprotection reagent which swells the resin maximally, has not been widely adopted. For example, the synthesis of a peptide recently described as "impossible"[43] was readily accomplished by the use of a 1% crosslinked resin and TFA-$CHCl_3$ (1:3) as the deprotection reagent[43a]. It is evident that for successful synthesis of large peptides or proteins, monitoring will be essential, both of deprotection[43,44], as well as coupling.

The coupling reaction in SPPS has been studied from the aspects of solvent[45], nature of the amino acid[46], chain length[47], adsorption of the amino acid derivative to the resin[48], and effect of triazole additives on coupling of active esters[49]. N-Ethoxycarbonyl-2-ethoxydihydroquinoline (EEDQ) has been investigated as a coupling agent[50], as well as a reversed SPPS using the azide coupling method[51]. The coupling of peptide fragments in SPPS may become extremely important for the synthesis of large molecules[50,52], especially with the use of a reagent such as hydroxybenzotriazole to reduce racemization[53], or of a system such as that of Wang and Merrifield[54] which gives protected peptide hydrazides directly from SPPS. Hydroxybenzotriazole is also useful for suppression of racemization of benzyl histidine in SPPS[55], although use of the Dnp or Tos derivative[56] would seem to be a better solution to that problem. Use of Na-NH_3 or hydrogenolysis for removal of benzyl groups from histidine cannot be recommended generally. A ninhydrin test for monitoring

completeness of coupling in SPPS has proved to be very popular[57]. Although the reliability of such monitoring techniques has been seriously questioned[58], some problems have been revealed by monitoring[59]. The consequences of incomplete reactions in SPPS, the so-called "failure sequences", have been discussed[60], and reagents have been described for terminating peptide chains where complete coupling cannot be achieved[61,62]. A rapid method for cleavage of amino acid residues from SPPS resins has been proposed[63].

Several tryptophan-containing peptides have been synthesized recently by SPPS[64-68]. Mercaptoethanol and 1,2-dimercaptoethane both appear to be satisfactory for protection of tryptophan in acidic reagents. Hydrolysis of peptides by p-toluenesulfonic acid in the presence of indole[99] allows direct analysis of tryptophan.

The synthesis of peptide amides is important for endocrinology. Recent reports describe cleavage of peptides from the standard SPPS resin by ammonolysis[69], or by transesterification to the methyl ester followed by ammonolysis in solution for C-terminal valine peptides (α-melanotropin, MSH)[70]. Another synthesis of MSH was achieved by conversion of the standard resin to a hydroxyphenyl resin derivative, from which the peptide could be removed by ammonolysis[68]. In this case racemization was a problem if the ammonolysis was done at room temperature. Benzhydryl-amine resins[71] offer much promise for the synthesis of amides, and have already been utilized in several syntheses[72,73].

The synthesis of peptides on soluble polymeric supports continues to receive some investigation[74,75], as well as the use of insoluble reagents[76,77] for synthesis of peptides in solution.

Synthesis of Specific Peptides - By far the most spectacular achievement of peptide synthesis is that of ribonuclease A by Gutte and Merrifield. This synthetic protein has now been purified to 80% specific activity[78]. Progress in improvement of the synthesis of ribonuclease S-protein by the classical method has been reviewed[79]. Synthetic studies[80] have revealed a very interesting peptide-protein recombination at the carboxyl end of ribonuclease similar to that of the S-peptide at the amino end. The synthesis of a 188-residue chain proposed for human growth hormone[81] was marred by the synthesis of the wrong sequence[82], and by the use of $Na-NH_3$, which probably cleaved the chain at Thr-Pro. The fact that some growth hormone and prolactin activity was obtained probably indicates that only portions of the chain are necessary for these activities. This has already been suggested by work on bovine growth hormone[83]. The synthesis of E. coli acyl carrier protein[58] appears to be very careful work, and high biological activity was obtained. Significant biological activity was also obtained in the synthesis of the basic pancreatic trypsin inhibitor peptide, but the synthetic methods used caused much damage to the native peptide[84]. In contrast, the synthesis of parathyroid hormone residues 1-34 gave a peptide with very high biological activity[35]. Careful synthetic studies on staphylococcal nuclease continue to appear from the laboratory of Anfinsen[85]. Details of

Katsoyannis' classical work on insulin have appeared[86] as well as continuing studies by SPPS on insulin from the laboratory of Weitzel[87]. Reports of new oxytocin analogs continue to appear. Particularly interesting are α-hydroxy oxytocin[88], which is more than three times as active as the parent hormone, and 4-threonine oxytocin[89], which has a very high ratio of oxytocic to antidiuretic activity. Several peptides from the C-terminus of cholecystokinin (CCK) have been synthesized, up to a tridecapeptide[90]. The smallest peptide possessing CCK activity is a heptapeptide. [8-Isoleucine]-angiotensin II seems to be a good inhibitor of angiotensin action[91], and the new approach of incorporating alkylating groups into peptide hormones[92] offers great promise for the future. Structure-activity relationships in the angiotensin and kinin fields have been reviewed[3,6,7]. Several syntheses of antibiotic peptides have appeared, notably those of gramicidin S[93,94], tyrocidins B[95], C[96] and E[97], and actinomycin D and analogs[98]. The potent hypotensive peptide, Substance P, has been characterized and synthesized[73]. For information on synthesis of hypothalamic and pituitary peptide hormones, the reader is referred to Chapter 18.

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Chapter 28. Preparation of Radioisotope-Labeled Drugs

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This report is concerned with the preparation of radioisotope-labeled drugs for use as drug tracers, not of radiopharmaceutical agents for use in diagnosis and therapy. Since this has not been the subject of previous reports in this series, particular attention will be given to general principles.

Literature Sources - Descriptions of the preparation of labeled drugs are remarkably few, scattered, and, to some extent, hidden in recent periodical literature. Since 1965 The Journal of Labelled Compounds has provided the greatest concentration of such publications. This journal also contains an abstracts section which provides additional information on synthesis with isotopes drawn from 40 periodicals as well as Chemical abstracts and Nuclear Science Abstracts. Otherwise, procedures for labeling drugs appear infrequently as such, or as part of a more comprehensive study, in a number of periodicals. A number of reference works[1-10] and proceedings of conferences[11-15], dealing in whole or in part with the preparation of labeled compounds, have appeared during the past 10 years. Several important works, notably those of Calvin[16], Murray and Williams[17, 18], Ronizo[19], and Kamen[20], although more than 10 years old, are useful sources of information.

Design of Synthesis - In using a radioisotope-labeled drug as a tracer one ordinarily is concerned with following the carbon skeleton of the drug molecule. To accomplish this, usually only one of the atoms in the molecule is labeled to serve as the tracer for the entire molecule. Since just the labeled atom is really being detected, it is important that this atom truly represent the carbon skeleton of the drug molecule. This concept should be considered when choosing a radioisotope and, particularly, when deciding on its location in the molecule. Possible changes the drug will undergo during the anticipated tracer study should be considered carefully. Carbon-14, in a suitable position, usually is satisfactory as a tracer for the carbon skeleton of a drug, but another radioisotope such as tritium, sulfur-35, or chlorine-36, serving as an auxiliary tracer for carbon, may be used to advantage. The actual choice of radioisotope involves a number of factors, including the molecular structure and intended use of the drug, the difficulty and expense of incorporating the isotope into the molecule, and the equipment available for detecting radioactivity. The maximum β energy (Mev), half life (years) and highest practical specific activity (mCi/m Atom), respectively, for the radioisotopes mentioned are: 0.155, 5570, and 40 for ^{14}C; 0.018, 12.5, and 25000 for ^{3}H; 0.167, 0.244, and 1000 for ^{35}S; and 0.714, 308000, and 0.3 for ^{36}Cl.

Since carbon and hydrogen are common to all drugs, carbon-14 and tritium usually satisfy the molecular structure requirements. Carbon-14 generally is preferred, since it is a more direct tracer of the drug's

carbon skeleton and is somewhat easier and more convenient to use. On the other hand, the need for high detection sensitivity, as with certain radioimmunoassays, may require a specific activity attainable only with tritium or sulfur-35. In autoradiography studies the higher energy of the carbon-14 β is desirable for whole-body section work whereas the weaker energy of the tritium β is necessary for high resolution at the cellular level. Chlorine-36 is not used often, because of its low specific activity. A potentially labile linkage between two parts of a drug's carbon skeleton occasionally necessitates double-labeling the drug. For this purpose it is desirable to label each part of the skeleton with a different isotope, such as carbon-14 and tritium, so they can be differentiated easily by radioactivity analysis during the tracer study. In practice each isotope usually is introduced in a completely separate synthesis so that two labeled drugs are obtained. This generally results in higher yields of each radioisotope and gives the user flexibility in selecting an isotope ratio for his double-label studies.

The total amount of labeled drug to prepare, in terms of radioactivity, depends on the anticipated studies, the radioisotope, and methods for detecting the radioactivity. In a typical drug-metabolism study involving 3 animal species and man, using liquid scintillation counting techniques, approximately one mCi of a carbon-14 or sulfur-35 labeled drug will suffice. Similar studies with tritium would require 2 to 4 mCi since the detection efficiency for tritium by the same techniques is only one-half to one-fourth that for carbon-14 and sulfur-35. On the other hand, the detection efficiency for tritium during whole-body autoradiography is only about one-fiftieth that of the two more energetic isotopes, so, if such studies are anticipated, even more tritium-labeled drug would have to be prepared.

The specific activity required for a labeled drug depends on the same 3 factors determining the total radioactivity required, and in addition depends on the anticipated drug dosage level in terms of weight. The more biologically potent drugs, such as steroid hormones, generally require higher specific activities than the less potent ones, such as antibiotics. A carbon-14 labeled drug having a specific activity of 10 mCi per mM can be quantified at a level of one nanogram per ml in body and excretory fluids by liquid-scintillation counting. A correspondingly higher specific activity would be necessary for a tritium-labeled drug. Care must be taken to prepare a labeled drug at a sufficiently high specific activity for its intended use; it can always be diluted with nonradioactive drug if necessary. However, too high a specific activity may make the synthesis unnecessarily difficult and will lead to increased radiation decomposition of the drug during storage.

The total amount of labeled drug to prepare, in terms of weight, will depend on the specific activity and the total amount of radioactivity required. This will vary widely but usually does not fall much below 0.5 mM for a drug prepared by a synthetic sequence. In the case of tritium exchange and reduction reactions it may be considerably lower, however.

Carbon-14 Labeling of Drugs - The most distinctive features of carbon-14 synthesis, as compared to regular organic synthesis, are: $^{14}CO_2$, the primary form of carbon-14, is the common starting material; high cost of the isotope; and need to work on a very small scale. Since $^{14}CO_2$ is the starting material, it first is necessary to convert it to a multi-carbon intermediate, either directly by carbonation of a Grignard or lithium reagent, or indirectly following its conversion to another single-carbon compound such as $K^{14}CN$, $H^{14}COOH$, $H_3{}^{14}COH$, or $H_2N^{14}CN$. These single-carbon intermediates, as well as a wide variety of multi-carbon intermediates, labeled with carbon-14 are available from suppliers. Whether to synthesize such an intermediate or purchase it from a supplier depends on the local expertise, the amount required, and the cost. The need for 25 to 50 mCi of a labeled intermediate might dictate its local synthesis, whereas one or two mCi might be more judiciously purchased. Whatever the source of the labeled intermediate it is converted to the desired drug by regular synthetic techniques, usually modified for improved yields and handling small amounts of material. Although a well-developed synthetic procedure for preparation of the nonradioactive drug usually is available, it may be of limited value as a route to the carbon-14 labeled drug. The synthetic route will be determined from a consideration of possible labeled intermediates, the desired location of the label in the carbon skeleton of the drug, and the usually small scale of the reactions.

The carbon-14 label should be introduced as late as possible in the synthetic sequence since the yield based on isotope usually is of most importance. The yield should be both good and reliable. To help assure this, nonradioactive "cold" runs should be conducted so that the radioactive "hot" run becomes routine. These cold runs should be made using the same scale, reagents, and equipment that will be used in the hot run. They should be carried through the entire synthetic sequence, not limited to a study of each step separately using pure shelf reagents. The judicious addition of nonradioactive carrier at various stages in the synthetic sequence is part of the strategy in carbon-14 synthesis. Addition of pure carrier serves to increase the chemical purity of a labeled intermediate in those cases where success of a reaction is dependent on a pure reactant. It also can be helpful for increasing radiochemical yields during recrystallizations and other solubility limiting operations. Although adding carrier increases chemical purity, it has no effect on radiochemical purity.

Biosynthetic incorporation of $^{14}CO_2$, although valuable for preparation of carbon-14 labeled endogenous materials, appears of limited utility for preparation of labeled drugs. Exceptions are the naturally occurring drugs such as digitoxin and naturally occurring compounds such as carbohydrates and amino acids that would be useful intermediates for chemical or biosynthetic preparation of labeled drugs. The biosynthetic conversion of labeled multi-carbon intermediates to labeled drugs or drug precursors by specific enzyme systems or growing organisms is of much more utility. For example, carbon-14 labeled prostaglandin E_1-2-^{14}C has been prepared by incubating 8,11,14-eicosatrienoic-2-^{14}C acid with sheep vesicular gland tissue[21]. The preparations of numerous carbon-14 labeled anti-

biotics by fermentation of labeled carbohydrates[22], amino acids[23], and fatty acids[24] have been described. A cheap carbon-14 labeled substrate for some fermentations might be the sugar syrup obtained by extraction of detached canna leaves following their photosynthetic incorporation of $^{14}CO_2$.

The most useful reference works dealing with the general procedures of carbon-14 synthesis are those of Calvin[16], Catch[1], and Ronzio[19]. Murray and Williams[17], written in the form of Organic Syntheses, and Schutte[3], containing an extremely comprehensive tabulation of carbon-14 synthesis through 1965, are of particular value. Less comprehensive treatments of carbon-14 synthesis are included in books by Kamen[20], Wang[6], Wilson[7], Chase and Rabinowitz[8], and Raaen[9]. Several conference proceedings contain specific papers dealing with carbon-14 synthesis[11,12,14,15].

Tritium Labeling of Drugs - Tritium can be incorporated effectively in drugs by three general methods: chemical synthesis, exchange reactions, and biosynthesis. One of the principal differences in preparing carbon-14 and tritium labeled drugs is that in the former case the isotope is introduced during formation of the carbon skeleton of the drug whereas in the latter case the isotope usually is added to the preformed skeleton. Consequently, incorporation of radioactivity in a drug is somewhat easier with tritium, but generally the carbon-14 labeled drug is superior as a tracer.

The monograph of Evans[2] is the single most comprehensive and useful volume dealing with preparation of tritium-labeled compounds. Other general references include books by Feinendegen[4], Schutte[3], and Murray and Williams[18], a review by Lee and Schmidt-Bleek[25], and a small brochure compiled by Evans[26]. The recent monograph by Thomas[10], dealing with deuterium, should provide valuable information for tritium labeling of drugs.

The method of choice for introducing tritium into a drug with high probability of success, at the highest specific activity, and in selected, or at least somewhat predictable, positions in the molecule is by chemical synthesis. Tritium gas, the primary form of tritium is the usual starting material although tritiated water is important also. Although tritium gas can be, and often is, used in the carrier-free state, tritiated water usually is not, primarily because of the high radiation density in such a condensed-phase system. Thus, for obtaining a drug of the highest specific activity, a method usually must be devised to introduce tritium gas directly into the drug or a synthetic precursor. Although most tracer uses of drugs do not require such high specific activities, increasing use of the radioimmuno method for drug analysis, the sensitivity of which depends on the specific activity of the labeled drug, is increasing the demand for the highest specific activities attainable.

Methods for direct introduction of tritium gas into a drug by synthesis generally involve catalytic hydrogenation, by either tritium addition to an unsaturated system[2,27,28] or tritium replacement of a halogen[2,29,30]. Such reactions are usually conducted on a micro or semi-

micro scale, at room temperature, and at atmospheric pressure. Catalysts such as platinum and palladium, sometimes supported on charcoal or calcium carbonate, are used. Particularly for high specific activities, nonpolar solvents are required since tritium gas undergoes catalytic exchange with hydroxyl and other labile hydrogens to dilute the tritium. If a polar solvent can not be avoided its volume should be small and the hydrogenation rate should be rapid since the exchange is usually slower than hydrogenation. Catalytic hydrogenation has been used successfully for tritium labeling a wide variety of compounds, particularly amino acids, steroids, fatty acids, and the purine and pyrimidine bases at high specific activities[2]. The main disadvantages of the method are the difficulty of obtaining a suitable precursor for hydrogenation and the possibility of a certain amount of nonspecific labeling, particularly with unsaturated precursors.

Tritium labeling by catalytic exchange of a compound's stable hydrogen with tritium of a solvent has been widely used for introducing tritium into a variety of drugs and other compounds[2]. In this method the compound, curie amounts of a tritiated solvent, and the catalyst are mixed and allowed to react, usually at an elevated temperature, for several hours to several days. The system can be homogeneous, as with acidic and basic catalysts, or heterogeneous, as with certain transition-metal catalysts. Homogeneous acid catalysis using tritiated sulfuric[31], phosphoric[32], perchloric[33], and trifluoroacetic acids[34], as well as aluminum chloride in tritiated water[35] and tritiated phosphoric acid-boron trifluoride complex[32] have been used[2]. They are generally suitable for labeling aromatic compounds, but often the conditions are so drastic that extensive decomposition of complex compounds occurs. Homogeneous basic catalysis in liquid tritioammonia[36] and in strongly basic media[37] has been reported but does not appear to be generally useful for labeling drugs.

Tritiation by heterogeneous catalysis using an activated transition-metal catalyst and a tritiated solvent, usually platinum and water or 70% acetic acid, at temperatures to 200°C has been widely employed[2,38-40]. It has been successful with a variety of structure types, particularly aromatic compounds, steroids, amino acids, and heterocyclic compounds such as purine and pyrimidine bases[2]. This usually is the method of choice for exchange labeling since it is rather generally applicable, gives products of high specific activity, requires only short exchange periods, and causes little radiation degradation. Its disadvantages are heat sensitivity of some compounds, low exchange rates for many aliphatic compounds, and catalyst poisoning. As with other solvent-exchange methods, the specific activity of the product is limited by that of the tritiated solvent.

The tritium gas-exposure method for radiation-induced exchange labeling of organic compounds was introduced by Wilzbach[41] in 1957 and has come to be known as the "Wilzbach method"[2,5,42]. In this method the compound to be labeled is exposed to curie amounts of carrier-free tritium gas in a sealed flask for a period of several days to several weeks. The activation energy necessary for exchange of stable hydrogen atoms in the compound with tritium gas is derived from the β-decay of tritium. Tritium

incorporation is directly proportional to the amount of tritium gas and the exposure time. For drug-type compounds incorporation of stably bound tritium is often 10 to 200 μCi per curie-day exposure[43-46]. By proper choice of the amount of drug exposed, such tritium incorporations provide specific activities sufficiently high for many, but not all, drug-tracer uses. The major deficiency with the Wilzbach method is extensive formation of tritiated by-products of very high specific activities. Although these by-products may represent only minor chemical impurities, they usually account for most of incorporated tritium. Since these impurities often closely resemble the desired product, high-resolution purification methods such as chromatography and counter-current distribution should be employed. Radiochemical purity must be established with great care for drugs labeled by the Wilzbach method.

Modifications of the Wilzbach method have been aimed at increasing the rate of tritium incorporation and decreasing the formation of impurities[2,5,42]. Three general areas have been investigated; activation of the system by external radiation sources, adsorption of the compound and tritium on charcoal, and use of a noble-metal catalyst. Radiation sources have included an electric discharge[43,47], ultraviolet light[48], microwaves[49,50], and γ- and x-rays[49]. These methods generally have been successful in increasing the rate of tritium incorporation but have not been particularly effective in decreasing the formation of high specific activity impurities. Adsorption of the compound and tritium gas on charcoal[51] appears to be a worthwhile modification of the Wilzbach method but has not been extensively studied for drug-type compounds. The third modification, that of exposing an intimate mixture of the compound and a catalyst, such as platinum or palladium black, to tritium gas[45,46,52] appears to be the most promising modification of the Wilzbach method. Incorporation of tritium usually is greater and production of impurities is no greater and often less, than by the conventional Wilzbach method.

Biosynthetic methods for direct introduction of tritium from a tritiated-water medium into a drug or intermediate can involve specific incorporation of tritium in the compound, using a particular enzyme system, or nonspecific incorporation, involving growth of an organism. Such methods have not been of great utility for preparing tritium-labeled drugs because of the large amounts of radioactive substrate which must be handled and the rather limited specific activities attainable. This appears to be inherent in the method since the labeled substrate, tritiated water, is the growth medium and therefore must be used in large excess. Also, the specific activity of the tritiated water must be sufficiently low to prevent radiation damage of the enzyme system or organism. Nevertheless an antibiotic has been prepared at low but adequate specific activity for metabolism studies[53]. The use of biosynthetic methods for converting tritiated organic intermediates, prepared by chemical or exchange methods, to labeled drugs is more useful. By this method drugs of relatively high specific activities can be obtained since substrates of high specific activities can be employed in relatively large amounts. Other antibiotics[54], several steroids[55], and prostaglandin E_2[56] have been prepared in this manner.

Purity of Labeled Drugs - Regardless of whether the labeled drug is prepared on the premises or by a supplier, the user should assure himself that it is sufficiently pure immediately prior to its use. Radiochemical purity is of primary importance, but chemical, radioisotopic, "radioisomeric," and pharmaceutical purities may be important also. The degree of radiochemical purity and the relevance of the other criteria of purity depend on the intended use of the labeled drug. Satisfactory radiochemical purity, that is, association of the radioactivity with the desired chemical form of the drug, nearly always must be greater than 95% and often exceeds 99%. Since most labeled drugs are prepared in relatively small amounts, especially when a high specific activity is necessary, critical establishment of chemical purity may be difficult or even impossible. However, nonradioactive impurities may accelerate decomposition of the drug[57,58], interfere with the usual biological or chemical action of the drug, and obscure the true specific activity of the labeled drug to the point that conversion of radioactivity levels to absolute drug-equivalent levels is meaningless. Thus chemical purity of a labeled drug, although often overlooked, can be important. Radioisotopic purity, that is, the association of the radioactivity of the labeled drug with the desired isotope, is not often a problem. There has been at least one report[59], however, of a carbon-14 labeled compound, obtained from a supplier, containing tritium, apparently in the same chemical form. Radioisomeric purity, that is, the association of the radioisotope with the specified location in the molecule, also is not often a problem. Particularly in the case of tritium-labeled drugs, however, positions in the molecule other than those specified may contain the isotope. Although this would cause no difficulty in many cases, such material could lead to erroneous conclusions in certain studies. Pharmaceutical purity of a radioisotope-labeled drug is a measure of its suitability and safety for use in humans. It should pass substantially the same chemical purity, sterility, and pyrogenicity tests required for the nonradioactive form of the drug.

Radiochemical purity is best determined directly by high-resolution separation processes, such as thin-layer, paper, gas-liquid, and column chromatography, electrophoresis, and countercurrent distribution, in which it is convenient to analyze for separation of components by monitoring for radioactivity (i.e., autoradiography, chromatogram scanning, eluate monitoring). In most cases several such systems should be employed; two or three well-chosen ones may suffice but there are cases where many more systems did not reveal a later-recognized radiochemical impurity. Another direct method for determining radiochemical purity is by reverse isotope-dilution analysis. In this method the labeled drug is diluted with a substantial amount of pure, nonradioactive-carrier drug and the material is purified (i.e., crystallization, chromatography). A resulting decrease in the specific activity (corrected for dilution) can be quantified in terms of the radiochemical purity of the undiluted labeled drug. This method is a valuable supplement to the chromatographic methods previously mentioned but is not nearly as sensitive. In some cases, particularly those involving a synthetic sequence, radiochemical purity can be inferred indirectly from the chemical purity of the labeled drug. This is totally

unjustified in the case of Wilzbach tritiations and where nonradioactive carriers have been added at various stages of a synthetic sequence.

Chemical purity of radioisotope-labeled drugs usually must be determined using analyses which require very small amounts of material or allow recovery of the analytical sample. This often can only be accomplished by working closely with the analyst. For example, the UV-absorption spectrum, specific activity, and chromatographic purity can be determined with a solution containing one mg or less of a drug. Although a relatively large sample is required for optical rotation measurements, most of it can be recovered. Radioisotopic purity of drugs labeled with the isotopes mentioned in this report usually can be determined by scintillation spectrometry and half-life measurement (if necessary). Ordinarily the presence of a foreign radioisotope would be due to contamination; it would thus constitute a radiochemical impurity and be detected as such. Radioisomeric purity of a labeled drug often can be inferred from the method of preparation. This is not always safe, however, particularly in the case of tritium. For example, unexpected intramolecular shifts and unexpected exchanges in the presence of hydrogen-transfer catalysts have been observed. Thus, if the position of the label is critical to the study, the user should assure himself of its location. This may require extensive degradation and derivatization procedures. A useful alternative, in the case of tritium, might be to carry out a trial synthesis with deuterium and determine the isotope distribution by NMR. Pharmaceutical purity of a radioisotope-labeled drug is determined primarily on the basis of its chemical purity, together with its sterility and freedom from pyrogens if intended for parenteral use.

The various purity requirements should be considered when planning synthesis of the labeled drug. The intended user should discuss this directly with the person or organization preparing the labeled drug. For example, chemical and radioisomeric purities may be assured only by the method of synthesis. Determination of chemical purity may be possible only by the supplier who has access to the bulk of the labeled drug. Brief discussions of purity requirements for labeled compounds are included in several of the general references[1-5,7]. In addition, an article by Peyser[60], a booklet by Catch[61], and a paper presented by Merrill and Lewis[59] are particularly relevant.

Stability and Storage of Labeled Drugs - Having assured himself of the purity of a radioisotope-labeled drug the user must try to maintain this purity. This is not an easy task since decomposition, due to self irradiation, ordinarily will be more pronounced than for the nonradioactive form of the drug. The first general observations of this effect were published by Tolbert, et al in 1953[62]. Since then several other excellent reviews and tabulations on the self-decomposition of radioisotope-labeled compounds have appeared[63], particularly from the group at The Radiochemical Centre in Amersham[64-67]. Other general discussions of the problem can be found in books by Feinendegen[4], Schutte[3], and Wang[6].

Although knowledge of self-induced radiation decomposition of radio-

active compounds is largely empirical, it is recognized to result from four basic factors. Bayly and Weigel[64] have classified these as primary (internal), primary (external), secondary, and chemical effects. The primary (internal) effect refers to transformation of the radioactive atom in a molecule to another element resulting in a chemically different molecule. Although the resulting molecule constitutes a chemical impurity, unless the daughter atom is radioactive or two radioactive atoms are in the same molecule, a radiochemical impurity will not be formed. Fortunately, since it can not be controlled, this effect is of little consequence in the case of radioisotope-labeled drugs. The primary (external) effect involves direct interaction of the radioactive particle with molecules of the compound to form impurities, both chemical and radiochemical. Appreciable decomposition results from this mechanism. The secondary effect refers to decomposition of the compound by chemically reactive species produced by the radioactive particle. These chemically reactive species may arise from any material within the range of the radioactive particle, not simply from the compound itself. This effect, although difficult to distinguish from the primary (external) effect, seems to account for most self-induced radiation decomposition. The chemical effect refers to decomposition unrelated to the radiation and would occur during storage of the nonradioactive form of the compound. Chemical decomposition may be more severe, however, in the case of a radioactive compound when it is prepared in such a small quantity that chemical purity is not assured and stored at a very low concentration in solution[57,58].

A compound's susceptibility to radiation is expressed in terms of a G(-M) value, that is, the number of molecules destroyed per 100 electron volts of radiation energy absorbed. Although the general relationship between the extent of decomposition and G(-M) is exponential, it is nearly linear when decomposition does not exceed 10%, and is directly proportional to the product of G(-M) and the amount of radiation energy absorbed. The latter value is the product of 4 factors: F, the fraction of the radiation absorbed; E, the average energy of the radiation; s_o, the specific activity of the compound; and t, the time (when it is short relative to the isotopes half life)[64]. In general this relationship holds for both primary (external) and secondary effects when F refers to the compound in the former case and to both compound and its immediate environment in the latter case.

Control of the three factors, t, s_o, and F is effective in limiting decomposition due to both primary (external) and secondary radiation effects by minimizing direct interaction of the radiation with the system. Since decomposition is time dependent, it can be minimized by keeping the time, t, between purification of a compound and its use as short as possible. Keeping the specific activity, s_o, of the compound at the lowest reasonable value also will minimize its radiation decomposition. The fraction of the radiation, F, absorbed by the compound can be minimized by dispersing or diluting the compound. Dispersing the compound as a thin film on the container wall or on paper or silica gel may allow the radiation to escape from the compound with minimum interaction. Although this is feasible for carbon-14 and sulfur-35, it is impracticable for tritium

due to its low radiation energy. Dilution of the labeled compound with nonradioactive compound, a solvent, or an inert material such as paper or silica gel serves to limit interaction of radiation with the labeled molecules by interposing inactive molecules in the path of the radiation.

Although dilution of the labeled compound with a solvent is an effective, and often necessary, means of limiting direct interaction of radiation with the compound, secondary effects due to production of chemically reactive species from the solvent may lead to extensive decomposition. Benzene is a good solvent since it does not produce high concentrations of reactive species. Water is particularly poor in this respect, although inclusion of a scavenger such as ethanol may retard formation of reactive species. Scavengers have not been found particularly useful in nonaqueous systems. In general a labeled compound, whether it is neat or diluted, should be stored at a low temperature, in the absence of light, and in an inert atmosphere to protect against secondary radiation, as well as purely chemical, decomposition.

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