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OPENING REMARKS

PIETRO PRATESI

Società Italiana di Scienze Farmaceutiche, Via Giorgio Jan, 18, Milan, Italy

This international Symposium on Medicinal Chemistry, organized by the Società Italiana di Scienze Farmaceutiche, follows 10 years after the first Symposium, organized by the same Society in Florence, and 4 years after the Münster Symposium. All three Symposia have been sponsored by the International Union of Pure and Applied Chemistry. The international chemical world's interest in Medicinal Chemistry is confirmed by the establishment, in 1970, of the Medicinal Chemistry Section of IUPAC.

Medicinal chemistry has been defined as a basic science of health; and indeed it has many facets, since it uses the theoretical and experimental means of all branches of chemistry, especially organic chemistry, physical chemistry and biological chemistry. For this reason each advance achieved in these branches of chemistry has a marked influence on medicinal chemistry, that is, on the latter's capacity to interpret the mechanism of action of drugs at the molecular level and to express qualitative and quantitative forecasts of the biological activity of substances.

While many useful drugs are still discovered by semiempirical methods, I think it can nonetheless be asserted that recent advances in medicinal chemistry in the theoretical field allow a more rational approach to drug design.

The great successes achieved on the various frontiers of chemistry in the last few years and the increased degree of contact between chemistry and the biological sciences have certainly not been foreign to this result.

It is for these reasons that the Organizers of this Symposium, in order to express the present trends in medicinal chemistry, have selected from among the most advanced topics some vivid examples of mutual cooperation among the basic sciences of medicinal chemistry. They felt that the main function of medicinal chemistry conferences is precisely to stimulate basic research in medicinal chemistry, to extend its frontiers and thus to encourage a better approach to therapeutic problems, in the interest of public health.

The scientific time-table of the Symposium is split into the following topics: (i) Biochemistry of microorganisms as a basis for the rational development of anti-infectious agents, (ii) Synthetic analogues of biochemical messengers, and (iii) Physicochemical properties and biological action.

Both main Lectures and Special Contributions were given. The latter are for the purpose of illustrating particular achievements that have been attained in the fields of research covered by the Symposium. Ample space was devoted to discussion, which was wide-ranging and lively.

The main Lectures and Special Contributions are published separately, the former in this issue of *Pure and Applied Chemistry*, the latter in a supple-

mentary volume that is being published by Butterworths of London at the same time.

The Organizers thank the International Union of Pure and Applied Chemistry for sponsoring the Symposium and the Associazione Industrie Chimico Farmaceutiche for having made it possible through generous financial support. The Organizers also wish to thank the scientists who have given their advice in the preparation of the Symposium, the lecturers, and the colleagues who have chaired the single sessions.

THE MECHANISMS OF SELECTIVITY AND ACTION OF PROTEIN SYNTHESIS INHIBITORS

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ABSTRACT

Systems for protein biosynthesis can be broadly classified, according to their mechanism and the selective action of drugs on their reactions, in two groups: the prokaryotic type (including bacterial, mitochondrial and chloroplasts systems) and the eukaryotic type (including cytoplasmic systems from mammals, higher plants, green algae, yeast, fungi and protozoa).

Inhibitors of protein synthesis are classified according to their specificity into those acting on (a) prokaryotic systems, (b) eukaryotic systems and (c) both prokaryotic and eukaryotic systems. Within this specificity they are further classified into those affecting (a) the small ribosome subunit, (b) the large ribosome subunit and (c) the supernatant factors. Taking into account the reactions in which they interfere, inhibitors of protein synthesis are classified into those inhibiting (a) the initiation phase, (b) the elongation phase and (c) the termination phase of protein synthesis.

THE MECHANISM OF PROTEIN SYNTHESIS

Translation of mRNA in protein takes place at the ribosome level. As shown in *Figure 1* the ribosome has two subunits which are separated after synthesis of the polypeptide chain is finished. We can distinguish along the ribosome, and including the subunits, two sites: the donor- or P-site and the acceptor- or A-site. The peptidyl transferase centre, which catalyzes peptide bond formation, is integrated into the structure of the larger ribosome subunit; part of this centre is on the P-site whereas another part is on the A-site. The overall reactions taking place in the biosynthesis of proteins by *E. coli* ribosomes according to the two entry sites translocation model is shown in *Figure 2*[1]. For the purpose of understanding the whole process, it can be divided in three phases: (a) *initiation*, (b) *elongation* and (c) *termination* (*Figure 2*).

The *initiation phase* starts with the initiator formyl-methionyl-tRNA coded by the initiator triplet AUG at the 5′ end of the mRNA and the order of nucleotide triplets in the 3′ direction determines the order in which subsequent aminoacyl-tRNA bind to the mRNA-ribosome complex. The anticodon region of tRNA is recognized and its interaction with mRNA

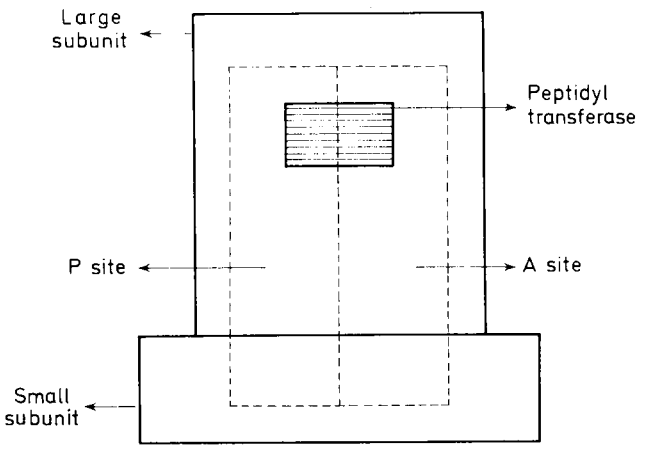

Figure 1. Ribosome structure.

specifically stabilized by the 30S subunit in a reaction requiring the initiation factors IF1, IF2, IF3 and GTP (*Figures 2a* and *2b*). In a further reaction a 50S ribosome subunit joins the mRNA–30S–f-Met-tRNA complex to complete the initiation complex with f-Met-tRNA bound to the donor- or P-site (*Figure 2c*). One GTP molecule is cleaved into GDP and Pi in the initiation phase; after the joining of the 50S subunit (*Figure 2c*). It is known that besides the codon–anticodon interaction at the level of the 30S ribosomal subunit, some portions of the tRNA, in particular the f-Met-bearing moiety, interact with the 50S subunit. The specificity for the initiating role of f-Met-$tRNA_F$ and its binding to the P-site is due to the α-NH_2 group of the methionine being blocked by formylation and to the unique structure of the $tRNA_F$.

In the *elongation phase* the aminoacyl-tRNA determined by the nucleotide triplet adjacent to the initiation codon is bound to the ribosomal acceptor- or A-site (*Figure* $2d_1$). Prior formation of the complex [elongation factor (EF) Tu–AA_1-$tRNA_1$–GTP] is required which in the binding reaction splits and EF Tu · GDP + Pi is separated. Once the f-Met-$tRNA_F$ is in the P-site and AA_1-$tRNA_1$ is bound to the A-site, *peptide bond formation* takes place catalyzed by the *peptidyl transferase* which is an integral part of the 50S ribosome subunit (*Figure* $2e_1$). Peptide bond formation takes place by transfer of the f-Met moiety in such a way that the —COOH group of methionine is linked to the α-NH_2 group of the amino acid AA_1-$tRNA_1$ bound to the A-site. The stripped $tRNA_F$ is then released from the P-site and the f-Met-AA_1-$tRNA_1$ moved to the P-site in a complex step known as *translocation* (*Figure* $2f_1$). The elongation factor EFG and GTP are required in this reaction and one molecule of GTP is cleaved to GDP + Pi. Movement of the mRNA in the direction $5' \rightarrow 3'$ is coupled to movement of the f-Met-AA_1-$tRNA_1$ from the A- to the P-site. Translocation results in the positioning of the next codon into site A which in turn allows entry and

specification of AA_2-$tTNA_2$ (*Figure 2d*$_2$) in a reaction similar to that of AA_1-$tRNA_1$ binding as described above. The ribosomal peptidyl transferase centre will then transfer the f-Met-AA_1-$tRNA_1$ to the A-site thus forming f-Met-AA_1-AA_2-$tRNA_2$. By repetition of the steps involved in the elongation cycle (aminoacyl-tRNA binding, peptide bond formation, translocation) the growth of the polypeptidyl-tRNA chain takes place with the polypeptide bound to the ribosome through the tRNA carrying the last amino acid incorporated into the chain.

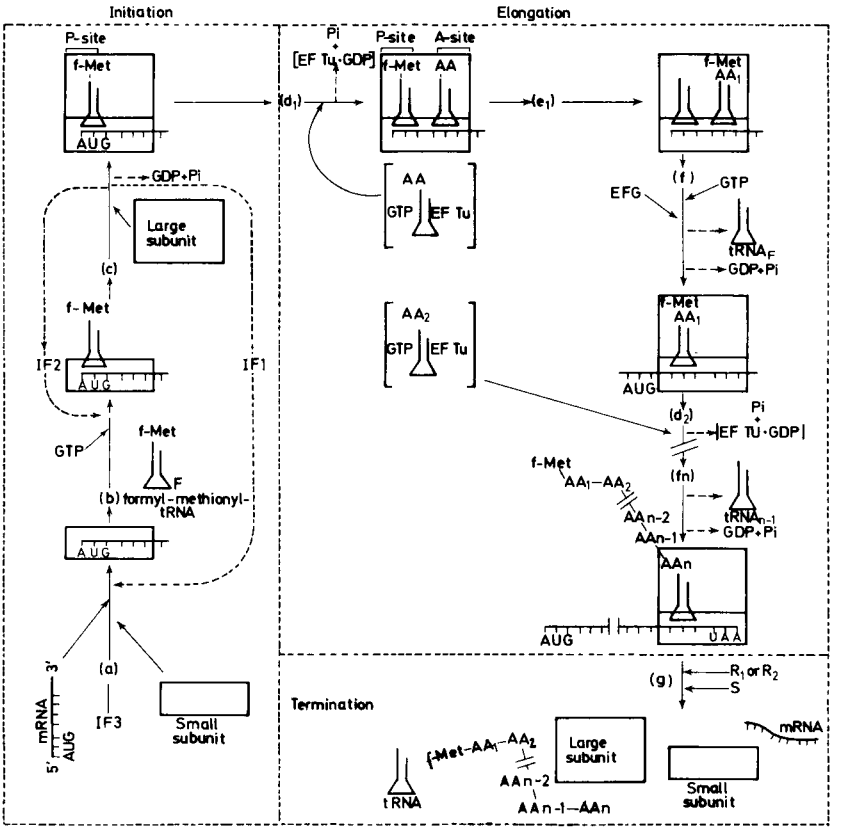

Figure 2. Protein synthesis by *E. coli* ribosomes.

For the *termination phase* a chain-terminating codon (nonsense codon) (either UAA or UAG or UGA) is recognized and the bond between the peptidyl and tRNA moieties of peptidyl-tRNA is cleaved in a reaction requiring the release factors (either R_1 or R_2) and the supernatant factor S (*Figure 2g*).

Basically the same mechanism for protein synthesis as in *E. coli* is found in organisms other than bacteria. However there are at least two broad

classes of systems for protein synthesis; one of them is the prokaryotic type (including bacteria, blue-green algae, mitochondria, chloroplasts and possibly nuclei) and the other one is the eukaryotic type (including systems from the cytoplasm of yeast, fungi, green algae, protozoa, higher plants and mammalian cells). Since bacterial ribosomes have a sedimentation coefficient of 70 svedbergs, ribosomes of the prokaryotic systems have been known frequently altogether as '70S type ribosomes' although it is now known that mitochondrial ribosomes are certainly smaller. Eukaryotic ribosomes are generally known as '80S type ribosomes' as their sedimentation coefficient is close to that figure. Bacterial ribosome subunits are 50S and 30S whereas those of eukaryotic ribosomes are 60S and 40S. The ribosomal subunits in eukaryotic type ribosomes perform similar functions to their prokaryotic counterparts, namely peptide bond formation on the larger subunit and codon–anticodon recognition in the smaller one (see *Figure 1*). However, functional differences between the prokaryotic and eukaryotic types of systems for protein synthesis are shown not only in the ribosomes but also in the initiator and in the different supernatant factors (initiation, elongation and release factors), since there is ample evidence that ribosomes and supernatant factors can be crossed between widely different systems belonging to the same type but not between systems of different types. The elongation factors EF-1 and EF-2 have been shown to have in eukaryotic systems a role rather similar to the bacterial factors EF-T and EF-G respectively; the release factor R has been shown to be required in eukaryotic systems and initiation factors have been isolated from eukaryotic cells but are not so well resolved yet as in bacterial systems. Furthermore some important differences have been shown within the prokaryotic type ribosomes since the 5S ribosomal RNA is known to be present in bacterial and chloroplast ribosomes but has never been found in mitochondrial ribosomes. Perhaps due to this difference no reconstitution of active ribosomes has been observed when the small subunit of mitochondrial ribosomes and the large subunit from bacterial ribosomes or *vice-versa* are mixed whereas there is reconstitution of active ribosomes when hybrid mixtures of chloroplasts and bacterial ribosome subunits are mixed. However for the purpose of the antibiotic action, we can consider, broadly speaking, all prokaryotic systems as a unit.

SELECTIVITY OF PROTEIN SYNTHESIS INHIBITORS

Most of the antibiotics known to block protein synthesis act at the ribosome level. Since there are at least two types of systems for protein synthesis, their inhibitors can be classified according to their specificity, into those affecting systems of (a) the prokaryotic type, (b) the eukaryotic type and (c) both the prokaryotic and the eukaryotic types (*Table 1*)[2–10]. Some of these inhibitors bind or affect directly either of the elongation factors (e.g. diphtheria toxin, fusidic acid and emetine) but most of them interact directly with the ribosome. There are still discrepancies regarding some of the results presented in *Table 1*. An important one concerns diphtheria toxin which is indicated in this *Table* as a specific inhibitor of elongation factor

Table 1. Inhibitors of protein synthesis

Acting on prokaryotic systems

Althiomycin
Berninamycin
Chloramphenicol group:
- Chloramphenicol
- D-AMP-3
- D-Thiomycetin
- D-Win-5094

Kasugamycin
Lincomycin group:
- Celesticetin
- Clindamycin
- Lincomycin (see Refs. 18, 19)

Macrolides group:
- Angolamycin
- Carbomycin
- Erythromycin (see Refs. 18, 19)
- Forocidin
- Lancamycin
- Leucomycin
- Methymycin
- Neospiramycin
- Oleandomycin
- Spiramycin
- Tylosin

Micrococcin
Multhiomycin
Siomycin group:
- Siomycin
- Sporangiomycin
- Thiopeptin
- Thiostrepton (Bryamycin)

Spectinomycin
Streptogramin A group:
- Ostreogrycin G
- Streptogramin A

Streptogramin B group:
- Staphylomycin S
- Streptogramin B
- Viridogrisein

Streptomycin group:
- Gentamycin
- Hygromycin B
- Kanamycin (see Refs. 18, 19)
- Neomycin (see Refs. 18, 19)
- Paromomycin (see Refs. 18, 19)
- Streptomycin

Viomycin

Acting on prokaryotic and eukaryotic systems

Actinobolin
Amicetin
Aurintricarboxylic acid
Blasticidin S
Bottromycin A_2
Chartreusin
Edeine A_1
Fusidic acid (see Refs. 16, 17)
Gougerotin
Nucleocidin
Pactamycin
Poly(dextran sulphate)
Poly(vinyl sulphate)
Puromycin
Sparsomycin
Tetracycline group:
- Chlortetracycline
- Doxycycline
- Oxytetracycline
- Tetracycline

Acting on eukaryotic systems

Anisomycin
Diphtheria toxin (see Refs. 10–15)
Emetine
Enomycin
Glutarimide group:
- Actiphenol
- Cycloheximide
- Streptimidone
- Streptovitacin A

Pederine
Phenomycin
Tenuazonic acid
Tylophora alkaloids:
- Cryptopleurine
- Tylocrebrine
- Tylophorine

Trichodermin
Tenuazonic acid

EF-2 of eukaryotic systems as accepted by most workers[11–14]: however, one group maintains that diphtheria toxin also binds specifically to the small subunit of bacterial ribosomes blocking the subsequent binding of

aminoacyl-tRNA[15–17]. Also in a few cases it appears that antibiotic sensitivity of systems of a given type differs within the different specific systems. For instance although EF-G from *Neurospora crassa* has been found to be interchangeable with bacterial EF-G[18], this factor from bacteria is sensitive to fusidic acid whereas the EF-G from *Neurospora* is not affected by this antibiotic[19]. Another interesting example is that of the antibiotics erythromycin, lincomycin, neomycin, kanamycin and streptomycin which have been shown to block protein synthesis by bacterial and yeast ribosomes but have been reported not to be active on ribosomes from mammalian mitochondria[20,21]. Two of the tylophora alkaloids (cryptopleurine and tylophorine) although included in *Table 1* as specific inhibitors of eukaryotic systems have also been reported to have a certain effect on yeast mitochondrial ribosomes[22]. Finally, not yet well-resolved cell-free systems from some mammalian structures have been reported to be sensitive to chloramphenicol and resistant to cycloheximide[23] contrarily to what might be predicted from the selectivity indicated in *Table 1*. However most of these apparently anomalous or exceptional results have only been reported by single groups of workers[15–23].

RIBOSOME SUBUNITS AS SPECIFIC TARGETS OF ANTIBIOTIC ACTION

The available methods to determine on which ribosome subunit a given antibiotic acts can be summarized as follows: (a) binding of radioactive antibiotics or competition with this binding; (b) reconstitution of hybrid ribosomes from ribosome subunits derived from antibiotic-sensitive and resistant cells, followed by studies on sensitivity of these reconstituted ribosomes to the required antibiotic; (c) studies on protein-synthesizing activity of ribosomes reconstituted from ribosome subunits pretreated independently with the required antibiotic followed by subsequent removal of the unbound inhibitor (by gel filtration, centrifugation, filtration or any other possible method) before the reconstitution experiments; (d) effects of antibiotics on a function specifically associated with a ribosome subunit which can be studied in the absence of the other subunit; and (e) it can be assumed that a number of antibiotics known to act on both bacterial and eukaryotic protein synthesis do so by blocking homologous steps in one or another case. Concerning point (d) indicated above we know at least three functions which can be carried out by the small ribosome subunit in the absence of the large subunit: (a) binding of mRNA, (b) formation of the complex aminoacyl-tRNA–small subunit-mRNA and (c) formation of the complex f-Met-tRNA–30S–AUG or natural mRNA. There are also a number of functions specifically catalyzed by the larger ribosome subunit: (a) peptide bond formation, (b) EF-T-dependent (in prokaryotic systems) and EF-1-dependent (in eukaryotic systems) GTP hydrolysis which normally takes place coupled to aminoacyl-tRNA binding to the ribosome and (c) EF-G-dependent (in prokaryotic systems) and EF-2-dependent (in eukaryotic systems) which normally takes place coupled to the complex step of translocation.

Table 2. Inhibitors of protein synthesis acting on prokaryotic ribosomes

Small subunit of action	
Aurintricarboxylic acid	Streptomycin group:
Edeine A_1	Gentamycin
Kasugamycin	Hygromycin B
Pactamycin	Kanamycin
Poly(dextran sulphate)	Neomycin
Poly(vinyl sulphate)	Paromomycin
Spectinomycin	Streptomycin
	Tetracycline group:
	Chlortetracycline
	Doxycycline
	Oxytetracycline
	Tetracycline
Larger subunit of action	
Actinobolin	Macrolides group:
Althiomycin	Angolamycin
Amicetin	Carbomycin
Blasticidin S	Erythromycin
Bottromycin A_2	Forocidin
Chloramphenicol group:	Lancamycin
Chloramphenicol	Leucomycin
D-AMP-3	Methymycin
D-Thiomycetin	Neospiramycin
D-Win-5094	Oleandomycin
	Spiramycin
	Tylosin
Fusidic acid	Puromycin
Gougerotin	Siomycin group:
Lincomycin group:	Siomycin
Celesticetin	Sporangiomycin
Clindamycin	Thiopeptin
Lincomycin	Thiostrepton
	Sparsomycin
	Streptogramin A group:
	Ostreogrycin G
	Streptogramin A
	Streptogramin B group:
	Staphylomycin S
	Streptogramin B
	Viridogrisein

By using a number of the experimental approaches indicated above it has been possible to elucidate the ribosome subunit in which some antibiotics act (*Tables 2* and *3*). Although fusidic acid is known to affect directly EF-G (in prokaryotic systems) and EF-2 (in eukaryotic systems) it is included in *Tables 2* and *3* as acting on the larger ribosome subunit since the antibiotic has been shown to bind forming the complex EF-G- or EF-2–larger ribosome subunit–GDP–fusidic acid[24, 25].

INHIBITORS OF THE INITIATION PHASE

Most of the inhibitors which are known to block the initiation phase of protein synthesis are shown in *Table 4*. Most of these compounds interact

with the smaller ribosome subunit. The antibiotic edeine A_1 and the compounds aurintricarboxylic acid, poly(dextran sulphate) and poly(vinyl sulphate) by binding to the smaller ribosome subunit of either prokaryotic or eukaryotic ribosomes block codon–anticodon interaction of the initiator f-Met-$tRNA_F$ and the initiation of protein synthesis is inhibited. Consequently the above inhibitors also block codon–anticodon interaction of peptidyl-tRNA of the growing chains at the level of the small subunit which is supposed to take place at the same place as the codon–anticodon interaction of the initiator; because of this, aurintricarboxylic acid, edeine A_1, poly(dextran sulphate) and poly(vinyl sulphate) are also inhibitors of the elongation phase. Furthermore these inhibitors have been shown to block equally well binding of f-Met-tRNA and aminoacyl-tRNA to the small

Table 3. Inhibitors of protein synthesis acting on eukaryotic ribosomes

Smaller subunit of action	
Aurintricarboxylic acid	Tetracycline group:
Edeine A_1	Chlortetracycline
Pactamycin	Doxycycline
Poly(dextran sulphate)	Oxytetracycline
Poly(vinyl sulphate)	Tetracycline
Sodium fluoride	
Larger subunit of action	
Actinobolin	Gougerotin
Amicetin	Puromycin
Anisomycin	Sparsomycin
Blasticidin S	Tylophora alkaloids:
Fusidic acid	Cryptopleurine
Glutarimide group:	Tylocrebrine
Actiphenol	Tylophorine
Cycloheximide	Trichodermin
Streptimidone	Tenuazonic acid
Streptovitacin A	Pederine

subunit. This might be considered a surprising result since the two-sites translocation model admits binding of donor and acceptor substrates taking place to different sites of the subunit (*Figures 1* and *2*); consequently data with the above inhibitors are presented by some workers as supporting a different variant of the translocation model with a single site of codon–anticodon interaction on the smaller subunit.

The antibiotics streptomycin, dihydrostreptomycin, kanamycin, gentamycin and paromomycin interact with the 30S subunit of prokaryotic ribosomes and do not appear to affect any of the steps 2a and 2b but destabilize the entire initiation complex formed in step 2c when the 50S subunit joins the f-Met-tRNA–mRNA–30S complex. This is probably a consequence of some distortion in the P-site since these antibiotics are also inhibitors of the elongation cycle by causing polysome breakdown.

Table 4. Inhibitors of the 'initiation phase'

Inhibitors of codon–anticodon interaction at the A- and P-sites of the small subunit (either 30S or 40S)	Inhibitors acting on the small subunit of prokaryotic ribosomes and destabilizing the initiation complex when the larger subunit joins the f-Met-tRNA-mRNA–30S subunit complex (*Figure 1c*)	Other inhibitors of initiation	Other possible inhibitors of the initiation phase
Aurintricarboxylic acid Edeine A_1 Poly(dextran sulphate) Poly(vinyl sulphate)	Streptomycin group: Dihydrostreptomycin Gentamycin Kanamycin Paromomycin Streptomycin	Pactamycin	Antibiotics blocking binding of the 3′ end of f-Met-tRNA to the P-site of the peptidyl-transferase centre of the larger subunit (see *Table 7*)

The main effect of the antibiotic pactamycin is located in the smaller subunit of either 70S or 80S ribosomes. By interacting with the small subunit pactamycin blocks formation of the complex f-Met-tRNA-30S-mRNA in bacterial systems and probably the equivalent reaction taking place in 80S type ribosomes.

A number of antibiotics have been shown to block peptide bond formation by interacting with the peptidyl transferase centre of the larger ribosome subunit. Some of these antibiotics have been shown in cell-free systems to block interaction of the CCA-Met-f- or CACCA-Leu-Ac-with the donor-site of the peptidyl transferase centre (*Table 7*). These inhibitors might be expected to block correct initiation of protein synthesis; in fact this mode of action has already been reported for some of these antibiotics.

INHIBITORS OF THE ELONGATION CYCLE

Inhibitors of aminoacyl-tRNA binding

The best known inhibitors of aminoacyl-tRNA binding to the ribosome are shown in *Table 5*. Included in this *Table* are edeine A_1, aurintricarboxylic acid, poly(dextran sulphate) and poly(vinyl sulphate) which, as indicated above, block interaction codon–anticodon to both A- and P-sites of the smaller subunit. On the other hand the tetracycline group of antibiotics specifically block codon–anticodon interaction at the A-site of the smaller subunit.

A number of antibiotics included in the siomycin group have been shown to block aminoacyl-tRNA binding to bacterial ribosomes at the level of the 50S subunit. Fusidic acid forms the complex fusidic acid–EF-G (or EF-2)–ribosome–GDP which prevents under certain conditions translocation (see *Table 8*) but also aminoacyl-tRNA binding to the larger ribosomal subunit of either bacterial or eukaryotic ribosomes. It is interesting to quote that fusidic acid has been reported to have no effect on *Neurospora* mitochondrial systems[19].

Some of the inhibitors of peptide bond formation have been shown to block binding of the terminal CCA-aminoacyl to the acceptor-site of the peptidyl transferase centre (*Table 7*) and might be considered not only as inhibitors of peptide bond formation but also as inhibitors of aminoacyl-tRNA binding at the level of the larger ribosomal subunits.

Inhibitors of peptide bond formation

The antibiotic puromycin is a structural analogue of the 3′-aminosyl-adenosine moiety of aminoacyl-tRNA; therefore puromycin acts on the A-site of the peptidyl transferase centre of prokaryotic and eukaryotic ribosomes forming a peptide bond with the initiator amino acid and blocking the correct peptide bond formation. Antibiotics of the chloramphenicol, streptogramin A, lincomycin and some macrolide antibiotics having the mycaminose-mycarose moiety have been shown to block peptide bond formation in most of the experimental systems from bacteria which have been devised (*Table 6*) although recent results in polysomal systems suggest that some of these antibiotics might not be proper inhibitors of peptide bond formation in intact bacteria[10].

Table 5. Inhibitors of aminoacyl-tRNA binding

Inhibitors of codon–anticodon interaction at the A- and P-sites of the smaller subunit (either 30S or 40S)	Inhibitors of codon–anticodon interaction at the A-site of the smaller subunit (either 30S or 40S)	Inhibitors of aminoacyl-tRNA interaction with the 50S ribosome subunit	Inhibitors of aminoacyl-tRNA interaction with the 50S and 60S ribosome subunits	Other possible inhibitors of aminoacyl-tRNA binding to the larger ribosome subunit
Aurintricarboxylic acid Edeine A_1 Poly(dextran sulphate) Poly(vinyl sulphate)	Tetracycline group: Chlortetracycline Doxycycline Oxytetracycline Tetracycline	Siomycin group: Siomycin Sporangiomycin Thiopeptin Thiostrepton	Fusidic acid	Antibiotic blocking binding of the 3′ end of aminoacyl-tRNA to the A-site of the peptidyl transferase centre (see *Table 7*)

Table 6. Inhibitors of peptide bond formation

Analogues of the aminoacyl-tRNA forming a peptide bond with substrates bound to the P-site	Blocking the peptidyl transferase centre of the 50S subunit	Blocking the peptidyl transferase centre of the 50S and 60S subunits	Blocking the peptidyl transferase centre of the 60S subunit
Puromycin	Chloramphenicol group: Chloramphenicol D-AMP-3 D-Thiomycetin D-Win-5094 Lincomycin group: Celesticetin Clindamycin Lincomycin Macrolides group: Angolamycin Carbomycin Spiramycin Streptogramin A group: Ostreogrycin G Streptogramin A	Actinobolin Amicetin Blasticidin S Gougerotin Sparsomycin	Anisomycin Trichodermin Tenuazonic acid

The antibiotics actinobolin, amicetin, blasticidin S, gougerotin and sparsomycin have been found to block peptide bond formation in prokaryotic and eukaryotic systems. However actinobolin is active in some of the systems and the activity of amicetin is very small in some of the ribosomes of the eukaryotic type tested. The antibiotic anisomycin is a very efficient inhibitor of peptide bond formation by ribosomes of the eukaryotic type.

For the reaction of peptide bond formation (*Figure* $2e_1$) the correct binding of the 3′ end of the substrates to the donor- and acceptor-sites of the peptidyl transferase centre is required. Studies on substrate binding have shown that some of the inhibitors of peptide bond formation block binding of the substrate to the acceptor-site of the peptidyl transferase centre whereas some others inhibit substrate binding to both donor- and acceptor-sites (*Table* 7).

Table 7. Inhibitors of substrate binding to the peptidyl transferase centre

	Inhibitors of CACCA-Leu-Ac binding to the P-site	Inhibitors of UACCA-Leu binding to the A-site
50S ribosome subunits	Lincomycin group: Clindamycin Lincomycin Macrolides group: Carbomycin Spiramycin Streptogramin A group Ostreogrycin G Streptogramin A	Chloramphenicol group: Chloramphenicol D-AMP-3 D-thiomycetin D-Win-5094 Lincomycin group: Clindamycin Lincomycin Macrolides group: Carbomycin Spiramycin Streptogramin A group: Ostreogrycin G Streptogramin A
60S ribosome subunits	Anisomycin	Anisomycin

Inhibitors of translocation

The step of translocation is one of the most complex and controversial in protein synthesis. Most of the systems used to study translocation are based on the extent of the puromycin reaction before and after treatment with elongation factor-G or -2 which is required for the GTP hydrolysis necessary for translocation. For this reason inhibitors of G-dependent GTP hydrolysis are usually considered as inhibitors of translocation.

Antibiotics of the siomycin group bind to the 50S subunit of bacterial ribosomes and block EF-G dependent GTP hydrolysis and the coupled translocation (*Table* 8). It is not well known where the site of EF-G interaction with the 50S ribosome subunit is located; however, since antibiotics of the siomycin group have also been shown to block aminoacyl-tRNA

binding to the 50S subunit (*Table 5*) the results suggest that the binding site for EF-G is overlapping the A-site of the 50S subunit.

Resistance to fusidic acid in bacteria is due to alterations in elongation factor-G, locating in this factor the action of the antibiotic. However it has been shown that fusidic acid is active in bacterial as well as eukaryotic systems allowing a single round of GTP hydrolysis and forming initially a stable complex fusidic acid–EF-G (or EF-2)–ribosome–GDP which prevents translocation and subsequent hydrolysis of GTP.

Table 8. Inhibitors of translocation

Acting on prokaryotic systems	Acting on prokaryotic and eukaryotic systems	Acting on eukaryotic systems
Siomycin group: Siomycin Sporangiomycin Thiopeptin Thiostrepton	Fusidic acid	Diphtheria toxin Pederine

Diphtheria toxin and pederine (a toxin from the insect *Paederus fuscipes*) have been shown to block translocation only by eukaryotic systems. However these toxins differ in their mode of action. Diphtheria toxin acts enzymatically in the presence of NAD and, by causing ADP-ribosylation of EF-2, blocks translocation. Pederine acts specifically on the ribosome but not on the elongation factors; by binding to ribosomes of eukaryotic cells pederine blocks the translocation and possibly some other steps in protein synthesis. Ribosomes of the insect producer of pederine are resistant to the toxin[26].

Other inhibitors of the elongation cycle.

All the inhibitors included in *Tables 5–8* obviously block the elongation cycle by preventing some of their partial reactions (aminoacyl-tRNA binding, peptide bond formation and translocation). However some other inhibitors of the elongation cycle have to be considered. Besides inhibiting the initiation phase and probably as a consequence of the same interaction with the ribosome the streptomycin group of aminoglycoside antibiotics are known to block in bacterial systems polypeptide elongation by causing polysome breakdown (*Table 9*).

There is evidence that a number of antibiotics are inhibitors of the elongation cycle but there is no clear indication of the specific reaction inhibited by some of them. By exclusion of other steps of protein synthesis most of these compounds are usually considered as inhibitors of translocation but certainly there is no clear positive indication in favour of this hypothesis. Among these inhibitors we can include a number of macrolides and antibiotics of the streptogramin B group (*Table 9*).

Table 9. Inhibitors of the elongation cycle other than those shown in *Tables 6–8*

Inhibitors causing breakdown of bacterial polysomes	Other inhibitors of the elongation cycle
Streptomycin group:	Macrolide group:
Dihydrostreptomycin	Chalcomycin
Gentamycin	Erythromycin
Kanamycin	Forocidin III
Paramomycin	Lancamycin
Streptomycin	Methymycin
	Neospiramycin III
	Oleandomycin
	Streptogramin B group:
	Staphylomycin S
	Streptogramin B
	Viridogrisein

Inhibitors of the termination phase

Peptide chain termination is a complex reaction requiring (a) recognition of the nonsense terminating codon, (b) peptidyl-tRNA hydrolysis which is catalyzed by the peptidyl transferase centre and (c) the release reaction specifically catalyzed by the release factors R_1, R_2 and S in bacteria and by the release factor R in mammalian systems. We do not know of any specific inhibitor of this step (c). Recognition of the termination codon UAG has been shown in bacterial systems to be inhibited by streptomycin and tetracycline (*Table 10*); as tetracycline also binds to 80S type ribosomes it is likely that this antibiotic also inhibits termination in eukaryotic systems.

Table 10. Inhibitors of the termination phase

Inhibitors of interaction of the nonsense terminating codon	Inhibitors of the release reaction
Streptomycin group	Inhibitors of peptide bond formation. (See *Table 6*)
Tetracycline group	

Peptidyl-tRNA hydrolysis required for peptide chain termination is known to involve the peptidyl esterase centre of the larger ribosome subunit in a reaction very similar in requirements and optimal conditions to that of peptide bond formation. It has been shown indeed that all peptide bond formation inhibitors tested (*Table 6*) are also inhibitors of the peptidyl-tRNA hydrolysis required for the termination reaction (*Table 10*); the specificities of the inhibition are similar as in the case of inhibition of peptide bond formation (*Table 6*).

REFERENCES

[1] J. Lucas-Lenard and F. Lipmann, *Ann. Rev. Microbiol.* **40**, 409 (1971).
[2] D. Vazquez and R. E. Monro, *Biochim. Biophys. Acta* **142**, 155 (1967).
[3] D. Vazquez and R. E. Monro, *Abhandl. Deut. Akad. Wiss. Berlin, Kl. Medizin*, Nr. 1, 569 (1968).
[4] D. Vazquez and R. E. Monro, *Agrochimica* **12**, 489 (1968).
[5] D. Vazquez, T. Staehelin, M. L. Celma, E. Battaner, R. Fernandez-Muñoz and R. E. Monro, in *Inhibitors, tools in cell research*, p. 100 (Ed. Th. Bücher and H. Sies), Springer-Verlag. Berlin–Heidelberg–New York (1969).
[6] D. Vazquez, T. Staehelin, M. L. Celma, E. Battaner, R. Fernandez-Muñoz and R. E. Monro, *FEBS Symposium* **21**, 109 (1970).
[7] B. Weisblum and J. Davies, *Bact. Rev.* **32**, 493 (1969).
[8] S. Pestka, *Ann. Rev. Microbiol*, **25**, 487 (1971).
[9] R. E. Monro, R. Fernandez-Muñoz, M. L. Celma and D. Vazquez, in *Drug action and drug resistance in bacteria*, p. 303 (Ed. S. Mitsuhashi). (1971) University of Tokyo Press (1971).
[10] Various contributions in *Molecular mechanisms of antibiotic action on protein biosynthesis and membranes* (Ed. E. Muñoz, F. García-Ferrandiz and D. Vazquez). Elsevier Publishing Co., Amsterdam (1972).
[11] R. J. Collier and J. A. Traugh, *Cold Spring Harb. Symp. Quant. Biol.* **34**, 589 (1969).
[12] D. M. Gill, A. M. Pappenheimer and J. B. Baseman, *Cold Spring Harb. Symp. Quant. Biol.* **34**, 595 (1969).
[13] T. Honjo, Y. Nishizuka and O. Hayaishi, *Cold Spring Harb. Symp. Quant. Biol.* **34**, 603 (1969).
[14] R. S. Goor and E. S. Maxwell, *Cold Spring Harb. Symp. Quant. Biol.* **34**, 609 (1969).
[15] N. Goto, I. Kato and H. Sato, *Japan. J. Exp. Med.* **38**, 185 (1968).
[16] A. Tsugawa, Y. Ohsumi and I. Kato, *J. Bact.* **104**, 152 (1970).
[17] I. Kato and A. Wattanuki, *J. South African Chem. Inst.* **22**, 125 (1969).
[18] M. Grandi and H. Küntzel, *FEBS Letters* **10**, 25 (1970).
[19] M. Grandi, A. Helms and H. Küntzel, *Biochem. Biophys. Res. Commun.* **44**, 864 (1971).
[20] F. C. Firkin and A. W. Linnane, *FEBS Letters* **2**, 330 (1969).
[21] P. J. Davey, J. M. Haslam and A. W. Linnane, *Arch. Biochem. Biophys.* **136**, 54 (1970).
[22] J. M. Haslam, P. J. Davey, A. W. Linnane and M. R. Atkinson, *Biochem. Biophys. Res. Commun.* **33**, 368 (1968).
[23] G. Ramirez, I. B. Levitan and E. Mushynski, *FEBS Letters* **21**, 17 (1972).
[24] J. W. Bodley and L. Lin, *Nature* **227**, (1970).
[25] J. W. Bodley, L. Lin, M. L. Salas and M. Tao, *FEBS Letters* **11**, 153 (1970).
[26] O. Tiboni, B. Parisi and O. Ciferri, *Giorn. Botanico Ital.* **102**, 337 (1968).

THE SIGNIFICANCE OF D-ALANYL-D-ALANINE TERMINI IN THE BIOSYNTHESIS OF BACTERIAL CELL WALLS AND THE ACTION OF PENICILLIN, VANCOMYCIN AND RISTOCETIN

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ABSTRACT

D-Alanyl-D-alanine is a key structure in the biosynthesis of the peptidoglycans of bacterial cell walls. It is introduced as the last step in the assembly of the precursor nucleotide compound containing muramic acid and remains throughout the biosynthetic process until the terminal D-alanine residue is lost at the final transpeptidation reaction required to effect crosslinking. This transpeptidation reaction is a target of penicillin action, and soluble carboxypeptidase–transpeptidases are inhibited by the antibiotic. The action of vancomycin and ristocetin is also tied up with the same D-alanyl-D-alanine terminus, but in a different way. These antibiotics contain an aglycone made up of phenolic amino acid residues in such a way that the resulting structure recognizes an acyl-D-alanyl-D-alanine terminus and combines with it with high affinity. By this mechanism bound vancomycin or ristocetin can inhibit reactions in the final stages of peptidoglycan synthesis. Correspondingly, in the presence of peptides that combine with vancomycin, the inhibition brought about by the antibiotic in either growing cells or in membrane preparations synthesizing peptidoglycan is reversed. At the same time some antibiotic remains bound to the preparations in such a way that it is no longer inhibitory. Studies with synthetic peptides have provided a rational basis for these observations.

INTRODUCTION

The cell walls of almost all bacteria contain a polymer known as peptidoglycan (mucopeptide, murein) that consists of polysaccharide chains substituted by peptides, some of which are crosslinked one to another[1, 2, 3]. The polysaccharide is a 1,4β-linked polymer of *N*-acetylglucosamine (exactly like chitin) except that each alternate residue has at C-3 an ether-linked D-lactic acid residue. The complete substituted hexosamine residue is called muramic acid. In some Mycobacteria the *N*-acetyl substituent on muramic acid is replaced by an *N*-glycolyl residue.[4] The muramic acid carboxyl groups are rarely free, except for a proportion of the residues in certain Micrococci, but are linked to peptide chains that are characteristic of

the peptidoglycan. The sequence of these amino acid residues in the precursor molecules, from which the peptidoglycan is biosynthesized, is shown in *Table 1*. The sequence of configuration is always L (or glycine)-D (γ-link)-L-D-alanyl-D-alanine. It is the presence of the terminal D-alanine dipeptide that is the main topic of this report.

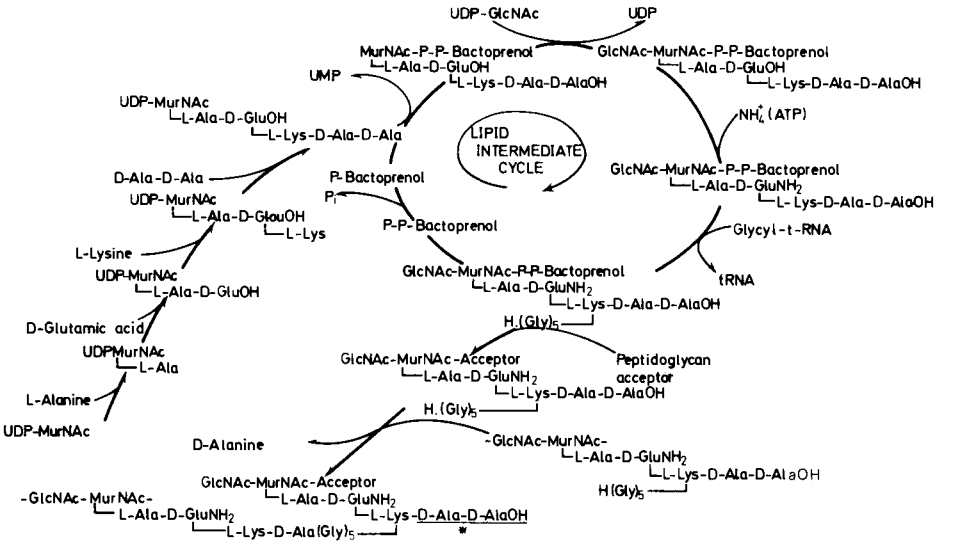
Table 1. Sequence of amino acid residues in the peptide portion of peptidoglycan precursors. The possible alternatives for each position are given, but not all combinations have so far been discovered.

Muramic acid → Aminoacid 1 →	Amino acid 2	γ-link →	Amino acid 3 →	Amino acid 4 →	Amino acid 5
L-alanine	D-glutamic		*meso*-diamino-	D-alanine	D-alanine
L-serine	acid		pimelic acid		
Glycine			(L-centre)		
			LL-diaminopimelic acid		
			L-lysine		
			L-ornithine		
			L-diaminobutyric acid		
			L-homoserine		
			L-glutamic acid		
			L-alanine		

The biosynthesis of the peptidoglycan, as at present understood, is shown in *Scheme 1*. It commences with the synthesis of the unique nucleotide precursor, specific for any particular organism, in which a UDP-*N*-acetylmuramylpentapeptide is built up. By the intervention of a lipid, bactoprenol[5], coupling to *N*-acetylglucosamine occurs and a lipid disaccharide-pentapeptide is assembled. Subsequently the crosslinking amino acids or peptides that occur in many organisms are added at the appropriate point on the primary peptide chain, e.g. in *Staphylococcus aureus* a pentaglycine unit is added to the ε-amino group of the L-lysine residue that occurs in the main chain[6]. Then the disaccharide units are polymerized to give glycan chains. After glycan synthesis a varying proportion of the D-alanyl-D-alanine termini are involved in a transpeptidation reaction, in which the terminal D-alanine residue is eliminated and the sub-terminal D-alanine residue forms a peptide bond with a free amino group on another chain. Depending upon the species, this free amino group may be at the D-centre of *meso*-diaminopimelic acid, the ω-amino group of L-lysine or L-ornithine, or the *N*-terminus of a crosslinking amino acid or peptide[2]. This crosslinking is considered to be essential for the stability of what Weidel and Pelzer called the 'murein sacculus'[7], the strong peptidoglycan network that surrounds the bacterium, the assumption being that by this means any newly synthesized glycan chain can be brought into covalent linkage with the rest of the network.

In many organisms, those D-alanyl-D-alanine termini that are not used for crosslinking as just outlined are then attacked by carboxypeptidases[8], either one or both residues being removed, so that in isolated cell-wall specimens the residual D-alanine is only that which is involved in crosslinks[9].

Scheme 1. Biosynthesis of cross-linked peptidoglycan in *Staphylococcus aureus**



* In some species, such as Bacilli, D-alanine residues in this situation are subsequently removed by carboxypeptidase.

The carboxypeptidase I attacks the terminal D,D linkage and removes a D-alanine residue, while carboxypeptidase II attacks an L,D linkage to remove the second D-alanine[10].

The foregoing account shows that the introduction and subsequent breakdown of the D-alanine dipeptide are key processes in the biosynthesis of the peptidoglycans of bacterial cell walls. It is hardly surprising, therefore, that these processes provide a target for the action of certain antibiotics. Those resembling D-cycloserine inhibit the enzymes involved in bringing the D-alanine dipeptide into the structure in the first place[11], but they are not our present concern. The crosslinking process and the removal of unwanted D-alanine residues are inhibited by penicillins on the one hand and by vancomycin and ristocetin on the other, by very different mechanisms, which we shall now consider further.

PENICILLIN

A connection between penicillin action and bacterial cell wall synthesis has been recognised for many years[12]. With the advent of cell-free systems synthesizing peptidoglycan, it became possible to study the system in detail. At first it was found that overall synthesis of peptidoglycan was not prevented by penicillin[13], except at very high concentrations, but later it became clear that in the presence of the antibiotic crosslinking was prevented[14]. Tipper and Strominger proposed a mechanism in which *N*-acyl-6-aminopenicillanic acid (penicillin) was supposed to be a structural analogue of *N*-acyl-D-alanyl-D-alanine[15]. By occupying a site on the transpeptidase that should have been

taken by the C-terminus of a peptide chain prior to crosslinking, the penicillin was thought to inhibit the enzyme action. Uncrosslinked peptidoglycan was therefore unable to link into the pre-existing network, so that faulty walls resulted. At the same time lytic enzymes, always present in the enzymic complex involved in wall synthesis and remodelling, might assist the death of the cell by breaking down parts of the wall that had been made prior to the addition of the antibiotic.

Izaki and Strominger[10] were able to show that *Escherichia coli* contained a carboxypeptidase I that was inhibited *in vitro* by penicillin, whereas carboxypeptidase II was unaffected. A further extension of the mode of action proposed by Strominger was that the transpeptidase, having accepted penicillin as a substrate analogue, should undergo penicilloylation so that it could no longer function even in the absence of penicillin. This conclusion was not supported by the results of Rogers, who found that *Staphylococcus aureus*, treated with penicillin and then washed free of external antibiotic, soon recovered the ability to synthesize peptidoglycan, even in the presence of chloroamphenicol that should have prevented the synthesis of new transpeptidase[16]. These ideas have been investigated extensively with penicillin-sensitive enzymes from *Streptomyces* species by Professor Ghuysen and ourselves. Covalent binding of penicillins to the *Streptomyces* carboxypeptidase–transpeptidase was not observed.

VANCOMYCIN AND RISTOCETIN

Both these antibiotics inhibit peptidoglycan synthesis and cause the accumulation of precursors in sensitive bacteria[17], and cell-free systems synthesizing peptidoglycan were 50 per cent inhibited by the same concentrations of vancomycin and ristocetin that were required for 50 per cent growth inhibition[18]. The formation of lipid intermediate (*Scheme 1*) was not inhibited, and was sometimes even enhanced, so that the antibiotic action had to occur at a later stage in peptidoglycan synthesis. The precise stage at which such inhibition might occur *in vivo* will be discussed after consideration of the probable chemical mechanism by which these antibiotics exert their action.

It was first observed in 1966 that vancomycin and ristocetin formed complexes with UDP-muramyl-pentapeptide precursors of peptidoglycan biosynthesis[19]. These complexes were formed *in vitro* and required the presence of the C-terminal D-alanine dipeptide; loss of even one of these D-alanine residues completely prevented complex formation[20]. Various alanine peptides were examined and it became clear that, first, complex formation was stoicheiometric, a given mass of antibiotic combining with one molecule of a suitable peptide and secondly, a D,D configuration and a free carboxyl group were essential for combination. Evidently, therefore, in the living cell vancomycin could combine with any of the precursors to which it had access, from the time of addition of the D-alanine dipeptide up to the final loss of one D-alanine residue during transpeptidation or by carboxypeptidase action. It was remarkable that a relatively small molecule like vancomycin should combine with such stereospecificity with a small peptide, and the system clearly merited further investigation. At this stage

relatively little was known about the chemistry of vancomycin, beyond the fact that it contained glucose, aspartic acid, *N*-methylleucine, phenols and chlorophenols[21].

For detailed study of the interaction we prepared an analogue of part of the uncrosslinked peptide chain of the peptidoglycan of *S. aureus* (or any other of the many organisms containing L-lysine at the central position in the pentapeptide), namely diacetyl-L-lysyl-D-alanyl-D-alanine. This substance combined very readily with vancomycin, thus facilitating physicochemical studies on the complex[22]. Vancomycin was titrated electrometrically and spectrophotometrically, and shown to contain groups with pK values of 2.9, 7.2, 8.6, 9.6, 10.5 and 11.7, the four last-mentioned being phenolic. The formation of the vancomycin–peptide complex under various conditions was studied by means of u.v. difference spectroscopy, since suitable peptides were known to produce a characteristic change in the absorption spectrum of vancomycin. In this way the association constant for the combination of antibiotic and peptide could be calculated. The stability of the complex in the range pH 1 to pH 13 indicated that the complex was formed only when carboxyl groups were ionized and phenolic groups were not, there being almost constant stability over the range pH 3 to pH 8, with a fairly sharp decrease on either side of those values. Furthermore, complex formation was not prevented by 8 M urea, 4 M KCl, 1 per cent sodium dodecyl sulphate or temperatures up to 60°C (*Table 2*). Although there was evidence that the

Table 2. Stability of the complex between vancomycin and diacetyl-L-lysyl-D-alanyl-D-alanine at pH 5 in the presence of various reagents.
Stability was calculated from the values for the association constant K_A, that in 0.02 M citrate buffer, pH 5.0 at 26°C without additions, being taken as 100%.

Substance added	Concentration	K_A (l mol^{-1})	Stability (%)
None	—	1.5×10^6	100
KCl	0.2 M	1.1×10^6	73
	2.0 M	1.8×10^5	12
	4.0 M	7.0×10^4	5
Urea	4.0 M	3.7×10^5	25
	8.0 M	5.0×10^4	3
Sodium dodecyl sulphate	0.10%	4.0×10^5	27
	0.38%	1.3×10^5	9
	0.95%	2.5×10^4	2
	9.50%	1.4×10^5	9

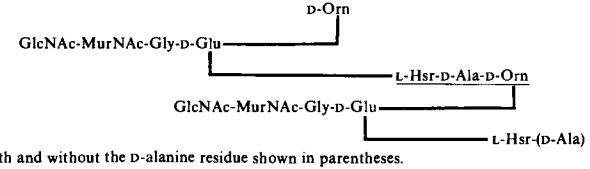
peptide carboxyl group was involved, the survival of the complex even in 4 M KCl seemed to weigh against a simple acid–base interaction being the main binding force. The results supported a minimum molecular weight for vancomycin of 1700–1800, but there were strong indications from optical rotatory dispersion and circular dichroism experiments that vancomycin molecules readily aggregated at higher concentrations, so that total aggregation was present at a concentration of 10 mg ml^{-1}. At the same time, the results suggested that vancomycin has only limited conformational flexibility.

We also studied the specificity of peptide structures that will form complexes

Table 3. Association constants and free enthalpy changes for the combination of vancomycin and ristocetin with peptides. α-Ac-L-Lys-D-Ala·D-Ala-NH_2 and Ac_2-L-Lys-D-Ala-Gly-NHOH did not combine with either antibiotic. Abbreviations: Aib, α-aminoisobutyric acid; MurNAc, *N*-acetylmuramic acid; Hsr, homoserine

Peptide	Ristocetin		Vancomycin	
	K_A(l mol^{-1})	ΔG(cal mol^{-1})	K_A(l mol^{-1})	ΔG(cal mol^{-1})
Changes in residue 1 (C-terminal)				
1. Ac_2-L-Lys-D-Ala-D-Ala	5.9×10^5	−7850	1.5×10^6	−8400
2. Ac_2-L-Lys-D-Ala-Gly	2.2×10^4	−5900	1.3×10^5	−6950
3. Ac_2-L-Lys-D-Ala-D-Leu	6.1×10^5	−7860	9.2×10^3	−5390
4. Ac_2-L-Lys-D-Ala-D-Lys	1.0×10^5	−6800	1.4×10^4	−5620
5. Ac_2-L-Lys-D-Ala-L-Ala	No combination		No combination	
6. Ac-D-Ala-D-Ala	7.2×10^4	−6600	2.0×10^4	−5840
7. Ac-D-Ala-Gly	1.9×10^3	−4470	5.4×10^3	−5070
Changes in residue 2				
8. Ac_2-L-Lys-Gly-D-Ala	1.6×10^5	−7070	9.4×10^4	−6760
9. Ac_2-L-Lys-D-Leu-D-Ala	5.8×10^4	−6470	2.9×10^5	−7420
10. Ac_2-L-Lys-L-Ala-D-Ala	No combination		No combination	
11. Ac_2-L-Lys-Aib-Gly	No combination		No combination	
12. Ac-Gly-D-Ala	4.9×10^4	−6390	1.1×10^4	−5500
Changes in residue 3				
13. Ac-Gly-D-Ala-D-Ala	1.6×10^5	−7070	9.4×10^4	−6760
14. Ac-L-Ala-D-Ala-D-Ala	2.2×10^5	−7270	3.1×10^5	−7450
15. *N*-Ac-L-Tyr-D-Ala-D-Ala	2.9×10^5	−7430	1.9×10^5	−7180
16. Ac-D-Ala-D-Ala-D-Ala	1.3×10^5	−6960	5.0×10^4	−6380
Influence of free amino groups				
17. L-Lys-D-Ala-D-Ala	8.2×10^3	−5320	1.2×10^4	−5510
18. α-Ac-L-Lys-D-Ala-D-Ala	1.9×10^5	−7200	4.7×10^5	−7700
Simultaneous changes in residues 1, 2 and 3				
19. Ac-L-Ala-Gly-Gly	2.5×10^3	−4620	4.9×10^3	−5200
20. Ac-Gly-Gly-Gly-Gly	8.0×10^2	−3950	1.5×10^3	−4300
21. Ac-L-Ala-D-Glu-Gly	6.8×10^2	−3850	4.8×10^5	−7720
22. *C. poinsettiae* dimer*	9.8×10^5	−8150	9.4×10^4	−6760

* The structure of this peptide is as follows:[26]



representing a mixture of molecules with and without the D-alanine residue shown in parentheses.

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with vancomycin and ristocetin, with the results summarized in *Table 3*[23, 24]. Certain similarities and differences emerged. Changes of amino acid at residue 1, in which the D-configuration was retained but the side-chain was increased from a methyl group (peptide 1) to more bulky groups (peptides 3 and 22) led to much lower affinity for vancomycin, whereas ristocetin continued to combine very well. Complete loss of carbon side-chain at residue 1 (glycine) led to a somewhat greater decrease in affinity for ristocetin than for vancomycin. Thus ristocetin imposes less exact steric restrictions for binding at position 1 of the peptide than does vancomycin. A positive charge on the side-chain at position 1 decreased combination with ristocetin but had the reverse effect with vancomycin (compare peptides 3 and 4). However, this latter enhancement of affinity was small compared with the deleterious effect for combination with vancomycin of a large side-chain at this position.

The effects of changes at residue 2 were particularly noticeable when the side chain was replaced by a hydrogen atom (peptides 1 and 8), causing considerable decrease in affinity for vancomycin but only a small change for ristocetin. On the other hand introducing a bulky side-chain decreased the affinity for ristocetin more than for vancomycin. With these results in mind, it is not surprising that peptide 21, with glycine at residue 1 and a bulky (and acidic) side chain at residue 2 (D-glutamic acid), should combine well with vancomycin but very poorly with ristocetin.

The fact that vancomycin will combine well with acetyl-L-alanyl-D-glutamyl glycine (peptide 21) and appreciably with the dimer from *Corynebacterium poinsettiae*, offers an explanation for another feature of the effect of the antibiotic on growing bacteria. It is known that vancomycin is rapidly absorbed by bacteria and that the isolated cell walls will take up considerable amounts[24]. Most peptidoglycans in fact contain, in addition to any remaining D-alanyl-D-alanine termini, other sequences that should combine with vancomycin or ristocetin, as can be seen by a comparison of the peptides in *Table 3* with known peptidoglycan structures. A few typical examples are given in *Table 4*. It is interesting that, as regards recognition of L-D-D carboxyl terminal sequences in addition to those ending in D-alanyl-D-alanine, vancomycin and ristocetin resemble the *Streptomyces* carboxypeptidases[26–28].

As shown above, the site on the vancomycin molecule that recognizes peptides contains phenolic groups. Similar groups are certainly present in ristocetin and ristomycin and other related antibiotics such as actinoidin[29], and the aglycones (deprived of their neutral sugars and also of the newly identified vancosamine[30] that may also be a common constituent of all these antibiotics), also act as antibiotics and combine *in vitro* with suitable peptides[24]. A partial structure was proposed for this aglycone, including a cyclic dipeptide composed of two diaminodicarboxylic acids, each consisting of two residues of hydroxylated phenylglycine joined by an ether link. Other hydroxyl groups were also present, so that all four aromatic rings retained pehnolic functions[31]. From this limited information, models could not be made with sufficient accuracy to clarify the mechanism of complex formation with peptides[24]. More chemical information will be needed before this problem can be finally solved.

Table 4. Some sequences found in crosslinked peptidoglycans and possible combination with vancomycin or ristocetin. The relevant residues are printed in italics.
N.B. Any D-alanine dipeptide termini that remain will also combine with both antibiotics.

Organism	Peptidoglycan structure	Proposed affinity for antibiotic	
		Vancomycin	Ristocetin
Micrococcus lysodeikticus	Disacc-L-*Ala*-D-*Glu*-*Gly* OH L-Lys-D-Ala-L-*Ala*-D-*Glu*-*Gly* OH L-Lys-D-Ala OH	Good	Poor
C. poinsettiae	Disacc-Gly-D-Glu L-*Hsr*-D-*Ala*-D-*Orn OH* Disacc-Gly-D-Glu L-Hsr-D-Ala OH	Quite good	Good
S. faecalis (fecium)	Disacc-L-Ala-D-Glu-NH_2 Disacc-L-Ala-D-Glu NH_2 L-Lys-D-Ala--- L-*Lys*-D-*Ala*-D-*Asp*-NH_2	None (terminates in amide)	None
B. megaterium KM	Disacc-L-Ala-D-Glu (L) D-Ala Disacc-L-Ala-D-Glu (Dap) (L) D-*Ala* OH (D) (Dap) OH (D)	Quite good	Good

COMPETITION FOR VANCOMYCIN BINDING SITES

In a cell-free system that was capable of peptidoglycan biosynthesis, the inhibitory action of vancomycin and ristocetin was reversed when large amounts of cell wall preparations that would absorb the antibiotics were added either simultaneously or after the inhibitory action had already manifested itself[32]. This reversal of vancomycin action was studied in more detail by the addition of solutions of a synthetic peptide that was also known to have affinity for the antibiotic[33]. Reversal of growth inhibition of *B. megaterium* was brought about by a molar ratio of peptide to antibiotic of 38, the time-lapse between addition of peptide and resumption of growth increasing with the time that the cells were left in contact with antibiotic alone. Similar results were observed with *S. aureus* and *M. lysodeikticus*. In parallel experiments a cell-free membrane preparation from *B. megaterium*, that could synthesize peptidoglycan *in vitro*, was also inhibited by vancomycin and again rapid reversal by added peptide was achieved. The fate of the antibiotic in these experiments was followed by the use of iodovancomycin labelled with ^{125}I[33, 34]. Experiments with growing cells and doses of iodovancomycin near the minimum inhibitory concentration showed that after growth inhibition had commenced some further uptake of antibiotic occurred, but the excess was released from the cells before rapid growth recommenced. The amount of antibiotic retained was about the same as when growth inhibition first occurred and hence it appeared that sensitive sites in the bacteria had been released from vancomycin inhibition. We concluded that perhaps in these limiting concentrations of vancomycin the antibiotic was being removed from inhibitory sites by the leakage of UDP-*N*-acetyl-muramylpentapeptide, with which it would combine. There was also considerable evidence from the experiments with whole cells and with membrane preparations that much of the antibiotic was sequestrated by being bound to sites that were not involved in biosynthesis. This conclusion fits admirably with the ideas on binding sites propounded in the previous section.

It seems likely, therefore, that in the living cell the vancomycin antibiotics will attach to any free D-alanine dipeptide termini that are available and by so doing will inhibit enzymes that bind to such fragments. Such an action was easily demonstrated with the soluble carboxypeptidase of *Streptomyces albus* G[35]. It must be borne in mind, however, that enzymes not immediately concerned with acting upon the D-alanine dipeptide itself will certainly have to recognize regions that contain it. Thus the enzyme that transfers a new disaccharide-pentapeptide unit from lipid-intermediate to preformed peptidoglycan may well have to recognize uncrosslinked pentapeptide side chains. If these side-chains were surrounded by vancomycin molecules recognition would be prevented, the acceptor could no longer be bound to the enzyme and chain extension could not occur. Proof of this hypothesis must await the unravelling of the complete enzyme chain of peptidoglycan synthesis.

CONCLUSION

D-Alanyl-D-alanine carboxyl termini are key structures in the biosynthesis of the crosslinked peptidoglycans of bacterial cell walls. Correspond-

ingly, antibiotics that either recognize and combine with these termini and thus interfere with biosynthesis (vancomycin and ristocetin) or those that inhibit enzymes, which in their turn recognize and modify the same termini (penicillins acting on carboxypeptidase–transpeptidases) are specific to bacteria and cannot react in the same way with host systems. Antibiotics of the vancomycin type that recognized only acyl-D-alanyl-Dalanine termini and showed low affinity for other structures in peptidoglycan should be effective at far lower concentrations than those presently known. A knowledge of the chemical structure of the binding site of vancomycin and ristocetin should go a long way on the path towards enabling such antibiotics to be synthesized. Consideration of the detailed effect of penicillins upon carboxypeptidase–transpeptidases, discussed in detail in Professor Ghuysen's paper[36], seems likely to throw new light both upon penicillin action and also upon the nature of the crosslinking reaction of peptidoglycan synthesis.

REFERENCES

[1] H. J. Rogers and H. R. Perkins, *Cell Walls and Membranes*, Spon, London (1968).
[2] J.-M. Ghuysen, *Bacteriol. Rev.* **32**, 425 (1968).
[3] M. J. Osborn, *Ann. Rev. Biochem.* **38**,501 (1969).
[4] J.-F. Petit, A. Adam, J. Wietzerbin-Falszpan, E. Lederer and J.-M. Ghuysen, *Biochem. Biophys. Res. Commun.* **35**, 478 (1969); A. Adam, J.-F. Petit, J. Wietzerbin-Falszpan, P. Sinay, D. W. Thomas and E. Lederer, *FEBS Lett.* **4**, 87 (1969).
[5] Y. Higashi, J. L. Strominger and C. C. Sweeley, *Proc. Nat. Acad. Sci. U.S.A.* **57**, 1878 (1967); K. J. I. Thorne and E. Kodicek, *Biochem. J.* **99**, 123 (1966).
[6] M. Matsuhashi, C. P. Dietrich and J. L. Strominger, *Proc. Nat. Acad. Sci. USA* **54**, 587 (1965).
[7] W. Weidel and H. Pelzer, *Adv. Enzymol.* **26**, 193 (1964).
[8] W. Leutgeb and W. Weidel, *Z. Naturf.* **18b**, 1065 (1963).
[9] R. C. Hughes, *Biochem. J.* **119**, 849 (1970).
[10] K. Izaki and J. L. Strominger, *J. Biol. Chem.* **243**, 11 (1968).
[11] J. L. Strominger, E. Ito and R. H. Threnn, *J. Am. Chem. Soc.* **82**, 998 (1960).
[12] J. T. Park and J. L. Strominger, *Science*, **125**, 99 (1957).
[13] A. N. Chatterjee and J. T. Park, *Proc. Nat. Acad. Sci. U.S.A.* **51**, 9 (1964).
[14] E. M. Wise and J. T. Park, *Proc. Nat. Acad. Sci. U.S.A.* **54**, 75 (1965).
[15] D. J. Tipper and J. L. Strominger, *Proc. Nat. Acad. Sci. U.S.A.* **54**, 1133 (1965).
[16] H. J. Rogers, *Biochem. J.* **103**, 90 (1967).
[17] P. E. Reynolds, *Biochim. Biophys. Acta* **52**, 403 (1961); C. H. Wallas and J. L. Strominger, *J. Biol. Chem.* **238**, 2264 (1963).
[18] J. S. Anderson, M. Matsuhashi, M. A. Haskin and J. L. Strominger, *Proc. Nat. Acad. Sci. U.S.A.* **53**, 881 (1965); *idem. J. Biol. Chem.* **242**, 3180 (1967).
[19] A. N. Chatterjee and H. R. Perkins, *Biochem. Biophys. Res. Commun.* **24**, 489 (1966).
[20] H. R. Perkins, *Biochem. J.* **111**, 195 (1969).
[21] F. J. Marshall, *J. Med. Chem.* **8**, 18 (1965).
[22] M. Nieto and H. R. Perkins, *Biochem. J.* **123**, 773 (1971).
[23] M. Nieto and H. R. Perkins, *Biochem. J.* **123**, 789 (1971).
[24] M. Nieto and H. R. Perkins, *Biochem. J.* **124**, 845 (1971).
[25] G. K. Best and N. N. Durham, *Arch. Biochem. Biophys.* **111**, 685 (1965); D. C. Jordan, *Can. J. Microbiol.* **11**, 390 (1965).
[26] M. Leyh-Bouille, J.-M. Ghuysen, R. Bonaly, M. Nieto, H. R. Perkins, K. M. Scheifer and O. Kandler, *Biochemistry* **9**, 2961 (1970).
[27] M. Leyh-Bouille, J. Coyette, J.-M. Ghuysen, J. Idczak, H. R. Perkins and M. Nieto, *Biochemistry* **10**, 2163 (1970).

[28] M. Leyh-Bouille, M. Nakel, J.-M. Frère, K. Johnson, J.-M. Ghuysen, M. Nieto and H. R. Perkins, *Biochemistry* **11** 1290 (1972).
[29] N. N. Lomakina, V. A. Zenkova, R. Bognar, F. Sztaricskai, Yu. N. Sheinker and K. F. Turchin, *Antibiotiki* **13**, 675 (1968).
[30] R. M. Smith, A. W. Johnson and R. D. Guthrie, *J.C.S. Chem. Comm.* 361 (1972); W. D. Weringa, D. H. Williams, J. Feeney, J. P. Brown and R. W. King, *J. Chem. Soc. Perk. Trans.* **1**, 443 (1972).
[31] N. N. Lomakina, R. Bognar, M. G. Brazhnikova, F. Sztaricskai and L. I. Muravyeva, *Abstr. 7th int. Symp. Chem. Natural Products, Riga* p. 625 (Ed. M. N. Kolosov), Zinatne, Riga (1970).
[32] R. K. Sinha and F. C. Neuhaus, *J. Bact.* **96**, 374 (1968).
[33] M. Nieto, H. R. Perkins and P. E. Reynolds, *Biochem. J.* **126**, 139 (1972).
[34] H. R. Perkins and M. Nieto, *Biochem. J.* **116**, 83 (1970).
[35] M. Leyh-Bouille, J.-M. Ghuysen, M. Nieto, H. R. Perkins, K. H. Schleifer and O. Kandler, *Biochemistry* **9**, 2971 (1970).
[36] J. M. Ghuysen, M. Leyh-Bouille, J. M. Frere, J. Dusart, K. Johnson, H. R. Perkins and M. Nieto, in *Medicinal Chemistry: Special Contributions–Milan 1972* (P. Pratesi, ed.), p. 1. Butterworths, London (1973).

INHIBITORS OF THE TRANSCRIBING ENZYMES

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ABSTRACT

The enzymes which transcribe DNA synthesizing RNA (DNA-dependent RNA polymerases) have structural differences in eukaryotic and prokaryotic cells, as indicated by the fact, among others, that there are substances which inhibit their function selectively in prokaryotic cells (streptolydigin and the ansa antibiotics, such as rifamycins and streptovaricin) and in eukaryotic cells (α-amanitin). Ansa antibiotics inhibit the initiation of RNA synthesis, whereas streptolydigin interferes with RNA elongation. Among ansa antibiotics, rifamycins have been studied more extensively, in order to obtain semisynthetic derivatives which, besides a comparable *in vitro* activity, showed better pharmacokinetic properties *in vivo*. Rifampicin, 3-(4-methylpiperazino-iminomethyl) rifamycin SV, has been selected for the oral treatment of various bacterial infections. Furthermore, a series of correlations between structure and activity have been derived, leading to the recognition of the essential structural requirements of the rifamycin molecule for penetrating into the bacterial cell and for inhibiting the enzyme.

Further chemical modifications have been performed, with the aim of overcoming the emergence of resistance in bacteria.

Members of the streptovaricin complex and some semisynthetic rifamycins have also been found to inhibit the RNA-dependent DNA polymerase (reverse transcriptase) of oncogenic RNA viruses. Their selectivity of action against the reverse transcriptase in respect to the polymerases of normal cells has yet to be established.

INTRODUCTION

Chemotherapeutic agents used in the treatment of infectious diseases possess, as a necessary but not sufficient requisite, a selective toxicity against pathogens. Therefore, they must act on specific targets which are present in the parasite but are either absent in the host or sufficiently different to be discriminable in the two organisms. In some cases, selective toxicity depends on the fact that the chemotherapeutic agent can reach the specific target in the microorganism but not in the host cell because of a selective permeability.

The search for chemotherapeutic agents has generally proceeded in an empirical way, namely, by means of a massive screening of products obtained by chemical synthesis or from natural sources, such as antibiotics, and through comparative tests of toxicity on the microorganism and host. So far this empirical approach has produced a series of useful chemotherapeutic agents that nowadays permit a successful control of most infectious diseases. Notwithstanding such successes, the necessity persists of carrying on the search for new chemotherapeutic agents with higher selectivity of action,

lower side-effects and an unchanged efficacy on the microorganisms resistant to current chemotherapeutic agents.

Probably, however, the search for new chemotherapeutic agents has reached a critical point. In fact, the number of new efficacious drugs produced in the last few years is very small although a systematic and massive effort has been maintained in the search for new synthetic compounds or microbial metabolites with antibacterial properties. On the other hand, the knowledge of cell structure and of comparative biochemistry has increased considerably. It has become clear that, besides the underlying unity of biochemical processes which occur in all forms of life, there are some differences in the biochemical pathways of diverse species. A knowledge of the structural or biochemical differences between prokaryotes and eukaryotes permits the selection of specific targets for new chemotherapeutic agents. It must be acknowledged frankly that, till now, such targets have been identified *a posteriori*. The discovery of chemotherapeutic agents has always preceded the explanation of their mechanism of action and, therefore, the characterization of the specific target on which they are directed. However, the interval of time elapsing between the discovery of a chemotherapeutic agent and the understanding of its mechanism of action is very short nowadays, tending to become an unitary process.

Presumably, the knowledge of biochemical processes in the various species may constitute, in the future, the basis for a rational development of new chemotherapeutic agents. It is clear that such a development does not only mean the ambitious possibility of designing *a priori* molecules suitable for hitting a particular target, that is the dream, too often not fulfilled, of each medicinal chemist. But the knowledge of particular targets allows the setting up of definite biological laboratory tests, fit for selecting products with a given mechanism of action. At the same time, this knowledge allows an orientation of the chemical or biochemical synthesis towards classes of products which, at least theoretically, could interfere with the chosen target.

A number of enzymatic systems having the same role but different structures in various species, have been discovered so far. They can be chosen as specific targets useful in the search for new chemotherapeutic agents. As a classical example, I would cite the dihydrofolate reductase enzyme. This enzyme catalyzes the synthesis of tetrahydrofolic acid, a cofactor of the C_1-transferase enzyme, which participates in the synthesis of purines and certain amino acids both in prokaryotes and eukaryotes. Dihydrofolate reductase is inhibited by a number of 2,4-diaminopyrimidines. The synthesis and testing of a large number of 2,4-diaminopyrimidines and related substances, revealed that considerable selectivity could be achieved through molecular modification of the inhibitor. Some substances in this group have found clinical applications. *Table 1* shows the selective action of pyrimethamine and trimethoprim against dihydrofolate reductase from different sources, and explains their use respectively as antimalarial and antibacterial agents[1].

Another class of specific targets is constituted by the transcriptases, enzymes which synthesize RNA using DNA as a template (DNA-dependent RNA polymerases). These enzymes have a analogous role, but a different structure, in prokaryotes and eukaryotes. This is shown by the fact that there are substances that selectively block their function either in eukaryotes (α-

Table 1. Comparative binding of diaminoheterocyclics by protozoal, mammalian and bacterial dihydrofolate reductase[1].

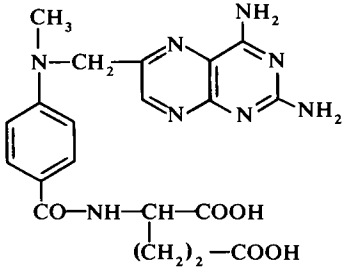
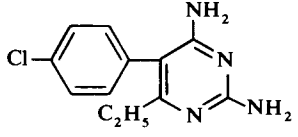
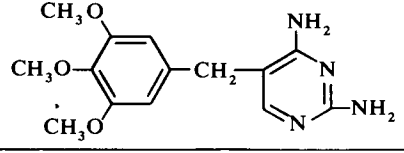
Compound	Structure	Concentration ($\times 10^{-8}$ M) for 50% inhibition				
		P. berghei	Mouse erythrocyte	Rat liver	*T. equiperdum*	*E. coli*
Methotrexate (Amethopterin)		0.07	N.D.	0.21	0.02	0.1
Pyrimethamine		ca. 0.05	100	70	20	250
Trimethoprim		7.0	>100000	26000	100	0.5

Table 2. Inhibitors of DNA-dependent RNA polymerase (DDRP)

	Bacteria	Nucleoplasma of eukaryotes
α-Amanitin	–	+
Streptolydigin	+	–
Most rifamycins and streptovaricins	+	–

amanitin) or in prokaryotes (streptolydigin and ansamycin antibiotics, such as the rifamycins and streptovaricins). *Table 2* shows the selective activity of these inhibitors. The discovery of such inhibitors has preceded the understanding of their mechanism of action, but the subsequent knowledge of this mechanism has catalyzed the search for analogous structures, obtainable, for instance, through chemical modifications of natural products, in order to obtain inhibitors of transcriptases in species others than sensitive bacteria. The results of this search, together with its prospects, are just the subjects of this report.

TRANSCRIPTASE (DNA-DEPENDENT RNA POLYMERASE): STRUCTURE AND FUNCTION

Transcriptase is the enzyme which catalyzes the polymerization of four ribonucleoside triphosphates into RNA, by transcribing a DNA template. In this way, the genetic information is transferred to RNA from DNA, with a sequence that is complementary to that of DNA template.

Eukaryotic transcriptase

The structure and the functioning of eukaryotic transcriptase is not well understood. At least three DNA-dependent RNA polymerases have been distinguished in eukaryotes. Polymerase I resides in the nucleolus and polymerase II and III in the nucleoplasm. They can be separated by chromatography on a DEAE-Sephadex column. Their reciprocal ratios seem to be different among the various species, and in the subsequent stages of development in the same species. Polymerase I synthesizes mainly, but not exclusively, ribosomal RNA; polymerase II synthesizes the bulk of nucleoplasmic RNA species, and polymerase III has no defined role as yet[2]. Recent studies on the structure of polymerase II from calf thymus and rat liver indicate that the molecule contains four components with molecular weight respectively of 190 000, 150 000, 35 000 and 25 000. Another species of polymerase II, with the largest component having a molecular weight of 170 000, has been identified in the same preparations. One of these forms may be derived from the other[3].

Prokaryotic transcriptase

This enzyme has been studied in several organisms. Most information has been obtained with the RNA polymerase from *Escherichia coli*. However, there are good grounds for believing that the structure and properties of this enzyme are very similar in the various kinds of bacteria.

Table 3. Composition of bacterial RNA polymerase

	Molecular weight
Complete enzyme at low salt concentration	990 000
Complete enzyme at high salt concentration	495 000
containing: 2 α-particles	40 000 each
1 β-particle	155 000
1 β′-particle	165 000
1 σ-factor	95 000

The RNA polymerase of *E. coli* is constituted of several subunits: two α, β,β′ and σ (see *Table 3*)[4]. All together, they constitute the holoenzyme, and the first four subunits constitute the core enzyme.

The enzymatic reaction occurs through the following steps:

(i) *Binding*. The enzyme (Enz) binds the template (DNA):

$$\text{DNA} + \text{Enz} \rightleftharpoons \text{DNA–Enz}$$

(ii) *Initiation*. At the initiation site, the DNA–Enz complex binds a purine nucleoside triphosphate which will constitute the 5′-terminal of the RNA chain, with the formation of a ternary complex:

$$\text{DNA–Enz} + \text{PPPPur} \rightleftharpoons \text{DNA–Enz–PPPPur}$$

A second nucleoside triphosphate (PPPX, X = purine or pyrimidine nucleoside) is then bound with the formation of the first phosphodiester bond and elimination of pyrophosphate (PPy):

$$\text{DNA–Enz–PPPPur} + \text{PPPX} \rightleftharpoons \text{DNA–Enz–PPPPur–PX} + \text{PPy}$$

(iii) *Polymerization*. The enzyme migrates on DNA, while the nucleoside monophosphates PX, complementary to those of the DNA template, are added to the 3′ end of the growing RNA chain:

$$\text{DNA–Enz–PPPPur–PX} + n_1\text{PPPX} \rightleftharpoons \text{DNA–Enz–PPPPur–(PX)}_n + n_1\text{PPy}$$

(iv) *Termination*. When the transcribing machinery reaches the end of a cistron or of a polycistronic message, the polymerization process stops and the DNA–Enz–RNA complex dissociates:

$$\text{DNA–Enz–PPPPur–(PX)}_n \rightleftharpoons \text{RNA} + \text{DNA} + \text{Enz}$$

The processes of initiation and termination are more complex than indicated in the above simplified scheme, because they require other factors regulating them. The σ unit has a role in the recognition and initiation of transcription of certain genes on DNA, but the process of chain elongation is determined by the core enzyme.

The structural and functional differences between RNA polymerase of prokaryotes and eukaryotes may constitute the basis for a rational development of chemotherapeutic drugs, acting on this specific target.

SPECIFIC INHIBITORS OF TRANSCRIPTASE

Specific inhibitors of eukaryotic transcriptase

α-*Amanitin.* α-Amanitin is a highly toxic cyclic octapeptide, isolated from the poisonous fungus *Amanita phalloides*[5] (*Figure 1*). It is a potent specific inhibitor of DNA-dependent RNA polymerase II of eukaryotes, while it does not inhibit nucleolar polymerase I and polymerase III of eukaryotes and

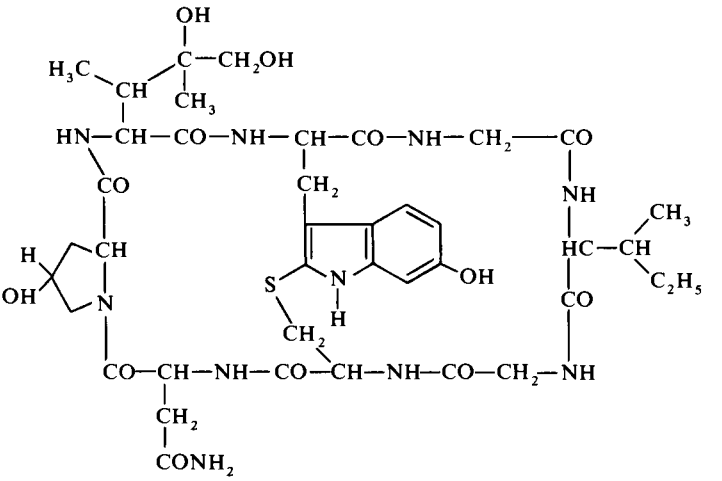


Figure 1. α-Amanitin.

bacterial RNA polymerase. As an illustration of the degree of specificity, it can be cited that nucleolar RNA polymerase II from rat liver is inhibited to the extent of 50 per cent at 10^{-8}M and to the extent of 100 per cent at 10^{-6}M of α-amanitin, while at the same concentrations, neither polymerase I and III of eukaryotes nor prokaryotic polymerase are inhibited to any significant extent[6,7]. The enzymatic reaction is blocked immediately after adding the inhibitor, which seems to act at the stage of RNA-chain elongation. The eukaryotic RNA polymerase from yeast is much less sensitive to the action of α-amanitin than the mammalian enzyme[8].

α-Amanitin can constitute a remarkable example of the possibility of finding substances with a selectivity of action on enzymes that, although having a similar role, have a different structure in the various species. Furthermore, its polypeptidic nature could constitute a suitable model for the synthesis and testing of analogous polypeptidic compounds, in order to obtain information concerning the part of the molecule of α-amanitin responsible for the binding to RNA polymerase II of eukaryotes. It is possible that, by introducing suitable groups such as aminoacidic residues, the molecule may acquire the property of binding to other polymerases.

Specific inhibitors of prokaryotic transcriptase

Streptolydigin. Streptolydigin is an antibiotic produced by *Streptomyces lydicus*[9]. Its structure is shown in *Figure 2*. It exhibits *in vitro* activity primarily against streptococci, diplococci and clostridia and is relatively nontoxic. It acts by binding and thus specifically inhibiting bacterial RNA polymerase. In contrast, it has no effect on polymerase from calf thymus[10]. Its binding to

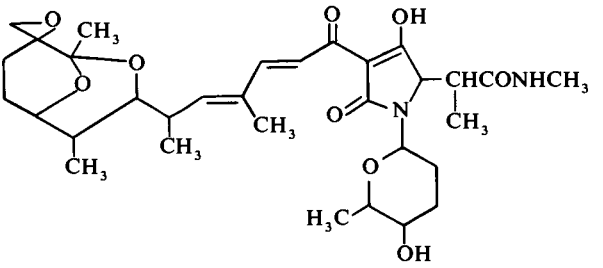


Figure 2. Streptolydigin.

the bacterial enzyme seems to be rather weak, since the inhibition is reversed by dilution. The concentration of antibiotic required for complete inhibition is fairly high (about 10^{-4}M), although a 50 per cent inhibition is obtained at about 7×10^{-6}M. Streptolydigin interferes with the process of RNA chain *elongation*, primarily by affecting the rate of phosphodiester bond formation[11]. Only at high concentrations of the drug is the initiation process affected, because the formation of the first phosphodiester bond is also inhibited.

Chemical modifications of streptolydigin have not been performed, so it is not possible to elucidate which groups of the molecule are responsible for the binding to the enzyme.

Streptolydigin has no clinical application, although it shows the requisite of a selective activity on prokaryotes. It is opportune to recall again the fact that the selectivity of action is an essential, but not sufficient requisite for a chemotherapeutic agent.

Rifamycins, tolypomycins and streptovaricins. Rifamycins, together with tolypomycins and streptovaricins, are natural ansa compounds in which an aliphatic bridge spans an aromatic system.

Rifamycins have been isolated from the fermentation broth of *Streptomyces mediterranei* as a complex of at least five antibiotics indicated as A, B, C, D, E[12,13]. The structure of rifamycin B (and of the related compounds rifamycin O, S and SV) (*Figure 3*) has been elucidated by chemical[14–16] and crystallographic[17] methods.

Other rifamycins have been isolated from the fermentation broths of *S. mediterranei* or its mutants: rifamycin Y[18,19], rifamycin L[20], 27-O-demethylrifamycin B, 27-O-demethylrifamycin SV and its deacetyl derivative[21]. Isolation of Streptomycetes or Nocardia strains producing rifamycin O has been reported by Japanese researchers[22,23]. A mutant able to produce directly rifamycin SV has been also obtained[24]. A list of all natural rifamycins is reported in *Figure 5*. Besides the natural rifamycins, several hundred deri-

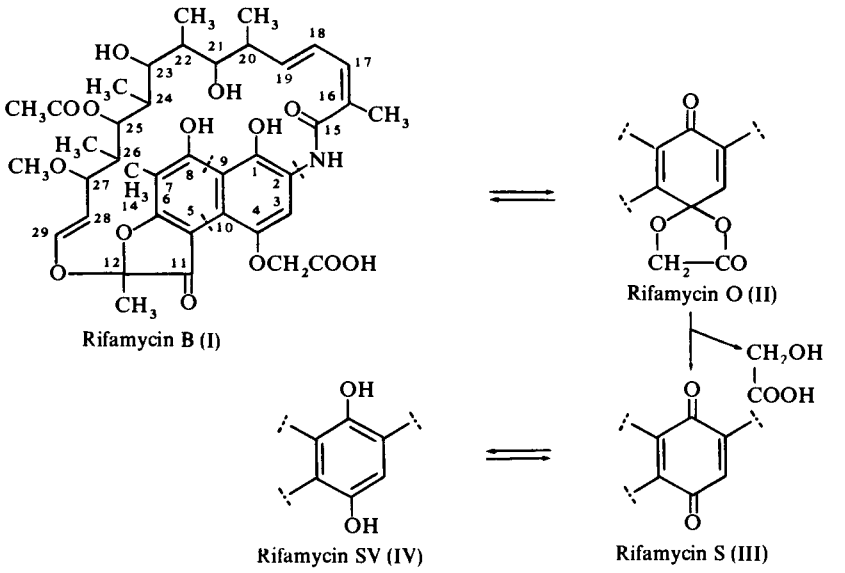


Figure 3. Structural relationship among rifamycin B, O, S and SV†.

vatives have been obtained through chemical modifications of rifamycin B, with the aim of obtaining products for therapeutic applications in the field of bacterial infections. These extensive studies have not only yielded three semisynthetic rifamycins now in clinical use (namely, rifamycin SV, rifamide and rifampicin) (*Figure 6*), but have led to the recognition of the main structural requirements for penetration into bacterial cell and for inhibition of the enzyme.

† The numbering system followed in this text for the rifamycins is that originally used by Prelog *et al.*[14–16] to identify the individual carbon atoms and their substituents. This numbering system has been used so far in all literature on the rifamycins and related 'ansamycins'. The orientation and numbering system for the rifamycins, according to the IUPAC rules, is shown in *Figure 4*.

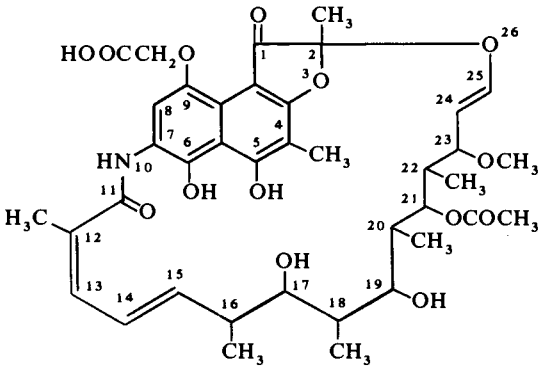


Figure 4. Numbering system for rifamycin B according to IUPAC rules.

Rifamycin-complex (A, B, C, D, E)
Rifamycin O
Rifamycin SV
27-O-Demethyl rifamycin B
27-O-Demethyl rifamycin SV
27-O-Demethyl-25-O-deacetyl rifamycin SV
Rifamycin L (4-O-glycolyl rifamycin SV)

Rifamycin Y

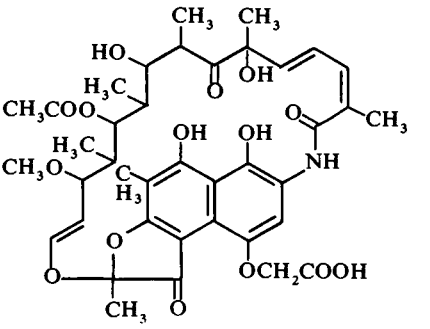


Figure 5. Natural rifamycins.

Tolypomycin (*Figure 7*) is an antibiotic substance produced by *S. tolypophorus*[25,26]. From its structure it can be considered a member of the rifamycin family, the main difference being the presence of the aminosugar, tolyposamine, in position 4. The product of mild acid hydrolysis, tolypomycinon, corresponds to rifamycin S with a methyl group and the adjacent double bond replaced by the cyclopropane ring and the carbonyl group, respectively.

Streptovaricin, produced by *S. mirabilis*[27], is a complex mixture of several related compounds whose structures are indicated in *Figure 8*[28,29].

Rifamycins, tolypomycins and streptovaricins are very active against Gram-positive bacteria and mycobacteria. Some members of this large group of natural and semi-synthetic antibiotics show also moderate activity against Gram-negative bacteria. Microorganisms resistant to one class of these antibiotics are also resistant to the others.

The mechanism of action on bacteria is identical for rifamycins, streptovaricins and tolypomycins. It will be referred to in detail here for the rifamycins, and in particular for rifampicin, which have been studied more extensively.

Rifamycins selectively inhibit the synthesis of all cellular RNA in sensitive bacteria[30], because they are potent inhibitors of the bacterial DNA-dependent RNA polymerase[31,32]. A concentration of 2×10^{-8} M of rifamycin causes a

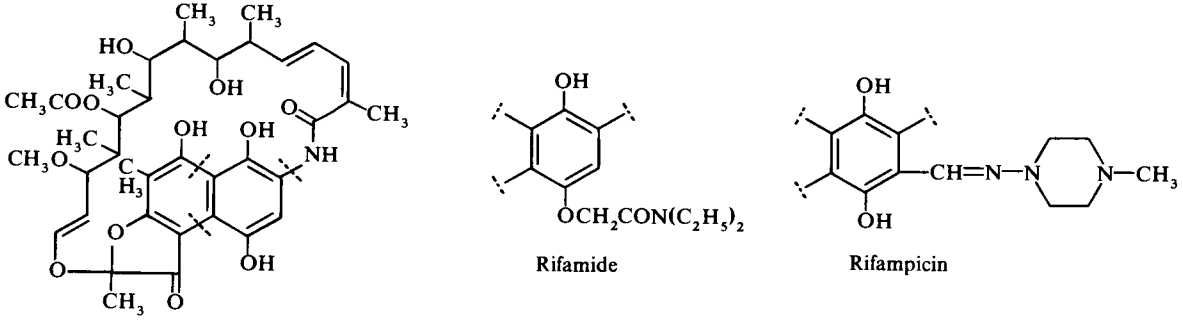


Figure 6. Rifamycins in clinical use.

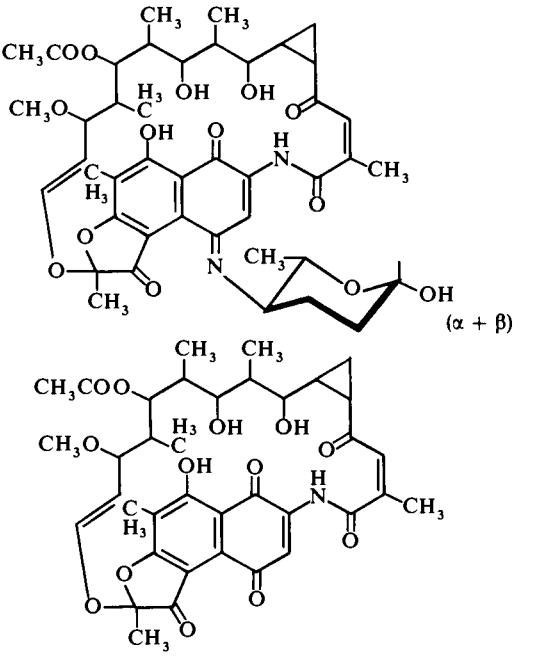
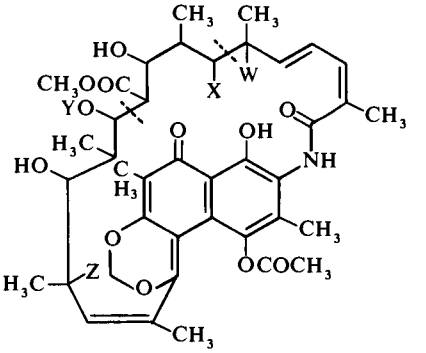
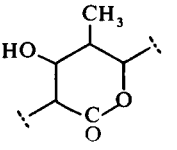


Figure 7. Tolypomycin Y (above) and tolypomycinon (below).



Streptovaricin	*W*	*X*	*Y*	*Z*
A	OH	OH	$COCH_3$	OH
B	H	OH	$COCH_3$	OH
C	H	OH	H	OH
D	H	OH	H	H
E	H	=O	H	OH
G	OH	OH	H	OH



F	OH	H	OH

Figure 8. Structural formulas of streptovaricins.

50 per cent decrease of the bacterial enzymatic activity. In intact bacteria the inhibition by rifamycins of protein synthesis and of DNA synthesis is a consequence of the primary effect of these antibiotics on RNA synthesis[33, 34]. The mammalian RNA polymerase is resistant to even very high concentrations of rifamycins[31] (*Figure 9*).

The high ratio of activity of rifamycins against bacterial and mammalian RNA polymerase could not be utilized for chemotherapeutic applications if these antibiotics inhibited the RNA polymerase of *mitochondria* of eukaryotic organisms, which have some biochemical properties similar to those of prokaryotes. Results of studies on the activity of rifamycins on mitochondrial RNA polymerase are conflicting. Some reports indicate that rifampicin does not inhibit the RNA synthesis by mitochondria from yeast[35, 36], *Neurospora*[37] and hamster cells[38], whereas those cases where rifampicin has been reported to affect mitochondrial-RNA synthesis, i.e. in rat liver or bovine heart[39–41], very high concentrations of antibiotics were needed for the inhibition.

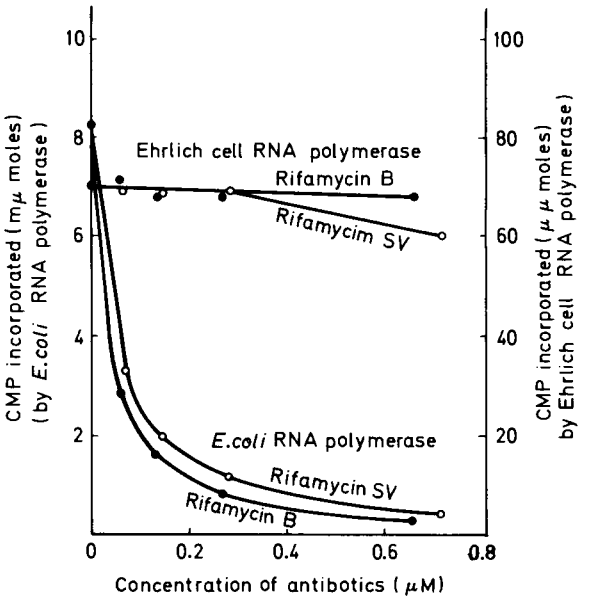


Figure 9. Effect of rifamycins on RNA polymerase reactions of *E. coli* and Ehrlich carcinoma cells[31].

In bacteria rifamycins inhibit the *initiation* of RNA synthesis and have no effect on chain elongation[31, 42]. They do not inhibit the formation of the enzyme–DNA complex, so their activity is probably due to their ability to modify the conformation of the enzyme, inactivating it before the incorporation of the first purine nucleotide of the RNA chain. As previous incubation of the holoenzyme with the natural DNA template in presence of Mg^{2+} gives protection against the inhibitory effect of rifamycins, it seems likely that these antibiotics inhibit the transformation of the DNA–enzyme

complex into an activated form, during which the enzyme binds to the specific promoter sites on the DNA[43–45]. Rifamycins inhibit the RNA polymerase forming a rather stable complex with it[46–48]. The binding between enzyme and rifamycins with the formation of an equimolecular complex is a very quick process[46]. The complex is rather stable but no covalent linkages are involved because it slowly exchanges with free rifamycin[48], and is dissociated with 6M guanidine hydrochloride[43]. Rifamycins also bind to the enzyme during the RNA chain elongation, but have no effect on this process, perhaps because the enzyme, at this stage, is resistant to conformational changes induced by the antibiotic.

Bacterial mutants resistant to rifamycins possess an altered RNA polymerase which is not inhibited by rifamycins[49, 50]. Studies on the interaction between rifamycins and the various subunits of the enzyme have shown that rifamycin binds to the β-subunit[51]. This has been confirmed by the finding that, in rifampicin-resistant mutants, RNA polymerase contains a subunit with an electrophoretic mobility different from that of wild-type β, likely as a consequence of the substitution of a single charged amino acid[52, 53].

STRUCTURAL MODIFICATIONS OF THE RIFAMYCINS AND THEIR EFFECT ON ANTIBACTERIAL ACTIVITY

Changes affecting penetration of rifamycins into the bacterial cell

With few exceptions, most rifamycins are active against bacteria when they are active against the bacterial RNA polymerase and *vice-versa* (*Table 4*). The exceptions, indicated as class 3 in the *Table*, are constituted by rifamycins bearing a strong polar group (e.g. a free carboxy group), which are active against the enzyme but have little or no activity against intact bacterial cells, because a permeability barrier exists, which the polar derivatives cannot pass[47, 54]. Such derivatives would be discarded, as inactive or little active, in a blind conventional screening for the search for antibacterial agents. On the contrary, a screening directed against the target enzyme, the bacterial RNA polymerase, would select such derivatives, leaving to the medicinal chemist the possibility of modifying their structure in order to increase their permeability through the cell wall. Also rifamycin B belongs to this group; but although inactive *per se*, it is easily transformed into rifamycin S, which is very active, and only as a result of this unusual property has not been neglected[55].

Table 4. Relationship between inhibition of bacterial growth and of bacterial RNA polymerase

Class	Activity of rifamycins	
	on bacterial cells	on bacterial RNA polymerase
1 Most active rifamycins	+	+
2 Most inactive rifamycins	–	–
3 Rifamycins unable to cross the bacterial cell wall	–	+
4 Rifamycins which undergo structural modification during antibacterial test	+	–

Although large variations exist in the sensitivity of different intact bacteria to a particular rifamycin, no such differences are apparent when the isolated transcriptase is examined. Thus in the case of the Gram-negative bacteria permeability plays an overriding role in determining the sensitivity to rifamycins; their enzyme has an intrinsic sensitivity very similar to that of the Gram-positive bacteria.

A few rifamycins show activity on the intact cells and no activity on the RNA polymerase and this is due to the fact that they undergo some chemical modification during the antibacterial test. For example 8-acyl rifamycins are inactive against the enzyme[54], but kill the bacteria because they undergo deacetylation during the incubation.

Structure–activity relationship for inhibition of transcriptase from sensitive bacteria

By making use of the large range of semi-synthetic rifamycins available, it has been possible to investigate the essential structural requirements of the rifamycin molecule for inhibition of the bacterial RNA polymerase. So far results have shown that changes of the ansa chain involving substitution or elimination of the two hydroxy groups at C-21 and C-23 yield inactive products, while the acetoxy group at C-25 and the methoxy group at C-27 seem to be unessential requirements. For example, methanolysis of rifamycin S in mildly acidic conditions, yields the 23, 27-epoxy derivative which no longer has the hydroxy group at C-23 and is inactive[47, 59]. Also the 21- and 23-O-acetyl rifamycins S have a very poor activity[58]. On the other hand, the acetoxy group at C-25 can be hydrolyzed without loss of activity[59]. Among natural rifamycins, the 27-O-demethyl rifamycin S shows a high level of activity, while rifamycin Y, with a keto group instead of an hydroxy group at C-21, is practically inactive[18]. It is interesting to point out that tolypomycin Y and streptovaricin A, C and D, which are very active, all have the hydroxy groups in positions corresponding to the 21 and 23 of the rifamycins.

Functional modifications which, although leaving the hydroxy groups at C-21 and C-23 unaltered, produce important changes in the conformation of the ansa chain, also give inactive or only moderately active products. Thus hexahydro rifamycin S shows very little antibacterial activity, while the dihydro and the tetrahydro analogues are quite active[56, 59]. The mono- and di-epoxides of rifamycin S, obtained by treatment with monoperphthalic acid, show a poor activity and the iminomethylether, obtained by treatment with CH_3I, has a negligible activity[56, 59] (see *Figure 10*). In all these cases, the molecular models and certain physicochemical characteristics indicate that such chemical changes have caused a modification in the conformation of the ansa chain.

The hydroxy group attached at C-8 of the chromophoric moiety also seems to be an essential structural requirement, since 8-methoxy rifamycin S is inactive[60]. On the contrary, the hydroxy groups on the positions C-1 and C-4 can be substituted by keto groups without loss of activity. The same quinone–hydroquinone system does not appear essential, since 4-deoxyrifamycin is also quite active, although its activity is approximately one-tenth that of rifamycin SV[56] (see *Figure 12*).

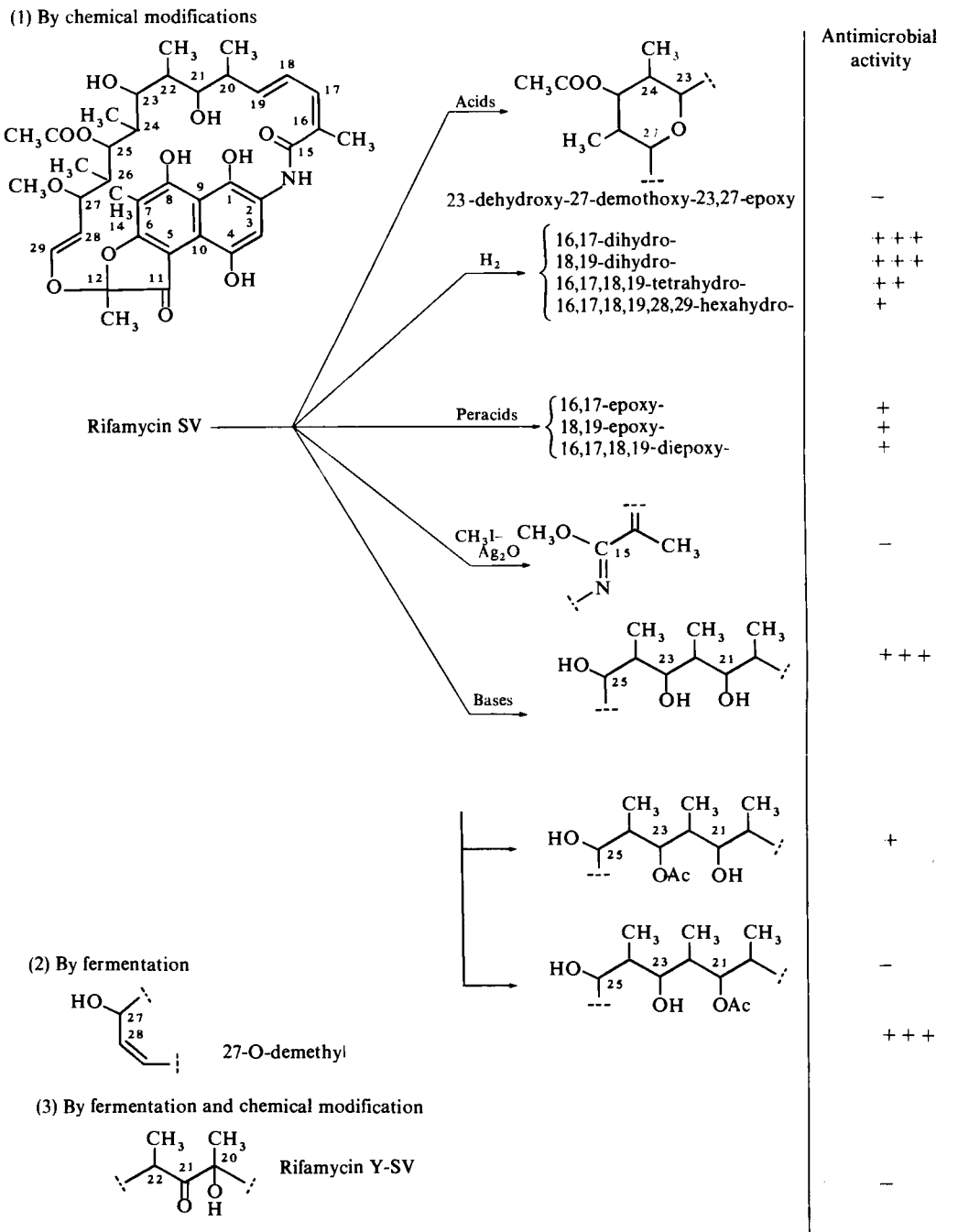


Figure 10. Modifications in the aliphatic ansa chain of rifamycin SV and related antimicrobial activity on *S. aureus*. (+ + + indicates m.i.c. of the order of < 0.02 μg ml^{-1}; + + 0.02–0.2; + > 0.2–2.0: – > 2.0 μg ml^{-1}).

Thus, from the data accumulated until now, it can be inferred that the essential structural requirements for the inhibition of the bacterial RNA polymerase, are free hydroxy groups at C-21, C-23 and C-8, together with a certain conformation of the ansa chain, leading to a definite geometric relationship between these groups, as can be seen in the three-dimensional model (*Figure 11*).

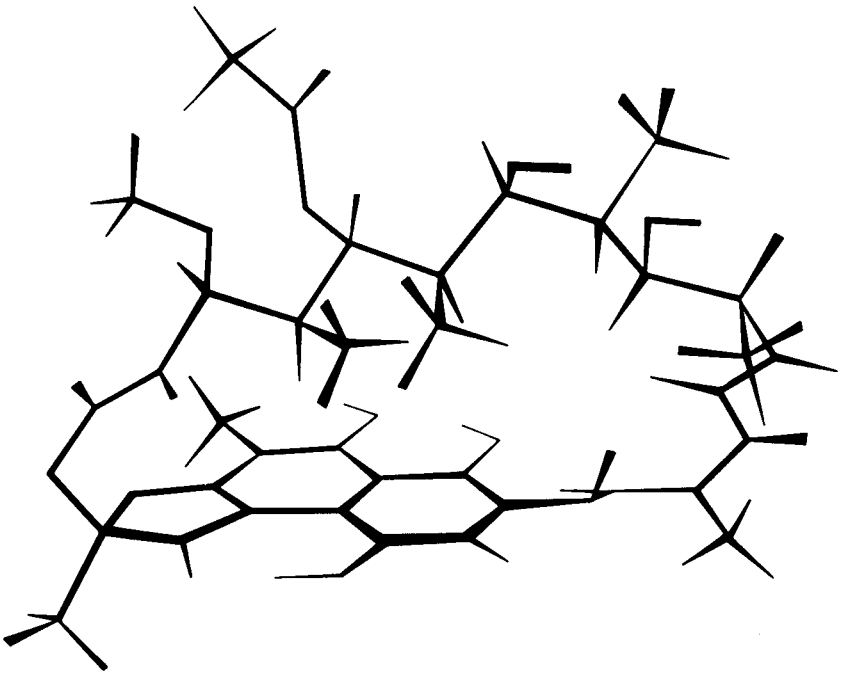


Figure 11. Conformational model of rifamycin SV.

Figures 12 and *13* indicate the principal classes of rifamycin derivatives with substitutions in position 3, 4, or both, studied in the course of several years: amides and hydrazides of rifamycin B[61]; quinonimino rifamycins[62,63]; 4-dialkylamino-4-deoxy-rifamycins[64]; phenazino- and phenoxazino-rifamycins[59,65]; pyrrolorifamycins[66]; 3-thioalkyl-[67], 3-dialkylamino-[59], 3-dialkylaminoalkyl-[68], 3-formyl-rifamycin SV and its functional derivatives[69,70]. The fact that substitutions in these positions with various substituents do not affect dramatically the antibacterial activity, indicates that this side of the molecule does not play an important role in the binding with the bacterial RNA polymerase.

Some differences have been observed in the *in vitro* antibacterial activity of the various classes of these derivatives, but they are most likely due to differences in the ability to penetrate into the bacterial cell, rather than to differences in the activity on RNA polymerase. As an example, *Table 5*

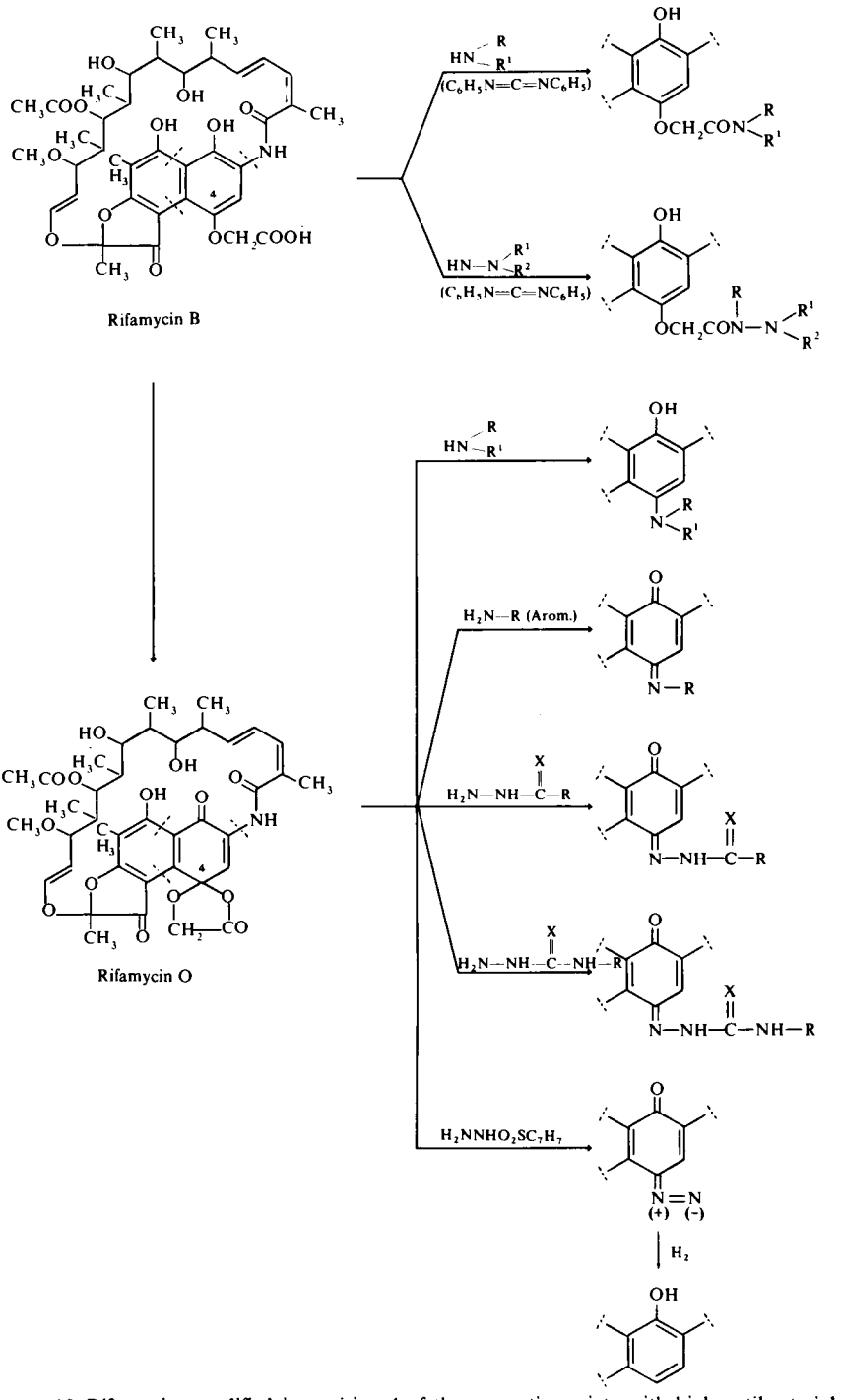


Figure 12. Rifamycins modified in position 4 of the aromatic moiety with high antibacterial activity (+ + +, according to *Figure 10.*)

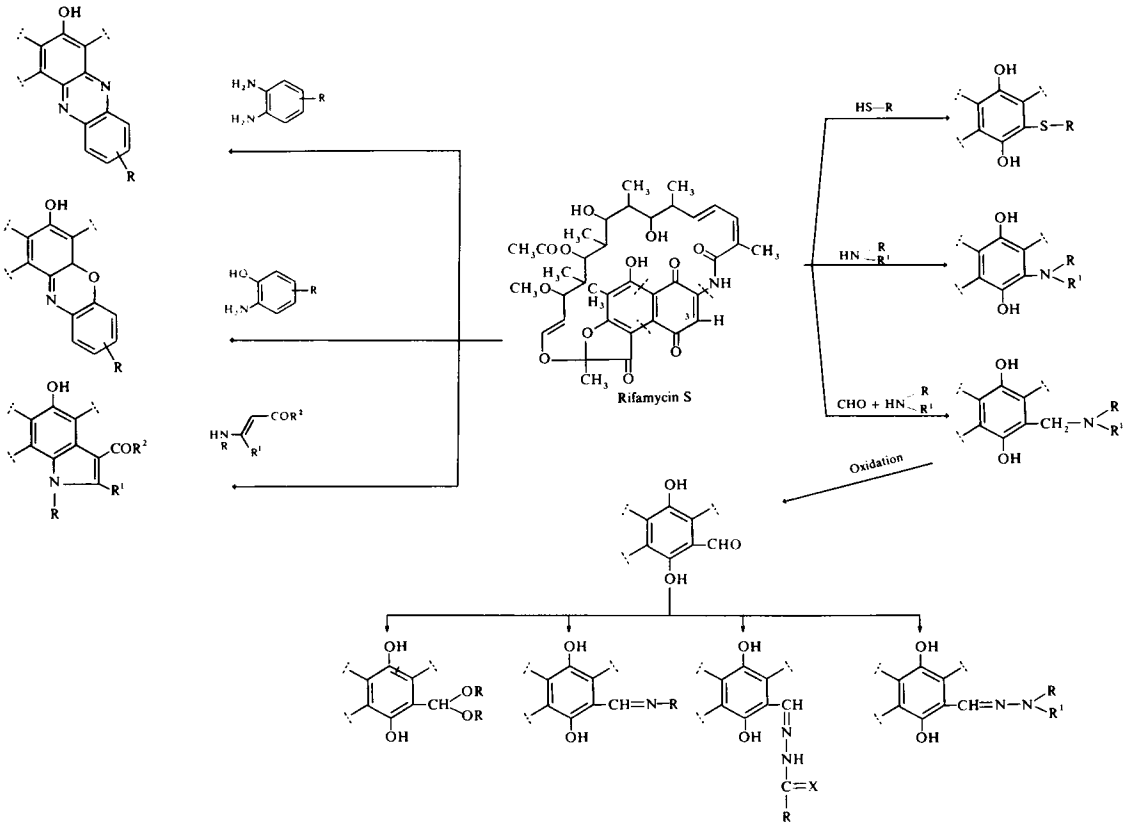
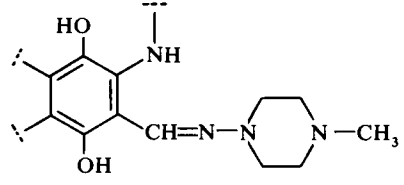
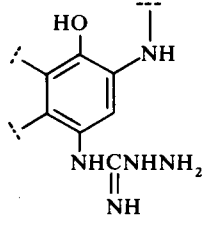


Figure 13. Rifamycins modified in position 3 and 3–4 of the aromatic moiety with high antibacterial activity (+ + +, according to *Figure 10.*)

Table 5. Rifampicin and rifamycin AG: antibacterial activity and inhibition of *E. coli* RNA polymerase

	Minimal inhibitory concentration ($\mu g\ ml^{-1}$)					% Residual activity of RNA polymerase at:	
	S. aureus	*Dipl. pneumoniae*	*E. coli*	*Ps. aeruginosa*	*M. tuberculosis*	0.1 $\mu g\ ml^{-1}$	1.0 $\mu g\ ml^{-1}$
Rifampicin 	0.002	0.01	10	10	0.5	52	2
Rifamycin AG 	0.002	0.001	0.5	1	0.001	63	5

reports the activity of rifampicin and rifamycin AG against several bacteria and against RNA polymerase. Although rifamycin AG is about 20 times more active than rifampicin against *E. coli*, the RNA polymerase extracted from this bacterial species is equally sensitive to both substances[60].

These chemical modifications on the aromatic moiety have produced dramatic changes on the pharmacokinetic behaviour of the resulting rifamycins in comparison with rifamycin SV, the first rifamycin used in therapy for the treatment of several infectious diseases. This modified pharmacokinetic behaviour is, in turn, the result of changes in physicochemical parameters (such as solubility and partition coefficient in water and lipids).

Rifampicin[69,71] has been selected out of several hundred derivatives for its high *in vivo* activity *per os* and is now successfully used for the oral treatment of Gram-positive and some Gram-negative infections and tuberculosis[72].

The field of rifamycins is another example showing that the selectivity of action against bacteria is a necessary requisite for the development of a potential chemotherapeutic agent, but that its practical usefulness depends on several other factors (absorption, distribution, rate of elimination, metabolism, interaction with proteins, etc.).

Modifications resulting in activity against rifampicin-resistant bacterial mutants

A possible target for new chemically modified rifamycins is the RNA polymerase of resistant bacteria, with the aim of overcoming the possible decline in the therapeutic value of these antibiotics as a consequence of the

Table 6. Activity of some rifamycin derivatives against sensitive and resistant strains of *S. aureus*

R	Rifampicin sensitive *S. aureus* m.i.c. (μg ml^{-1})	Rifampicin sensitive *S. aureus* Activity on DNA-dependent RNA polymerase	Rifampicin resistant *S. aureus* m.i.c. (μg ml^{-1})	Rifampicin resistant *S. aureus* Activity on DNA-dependent RNA polymerase
—CH=N—N⟨ ⟩N—CH$_3$ (rifampicin)	0·002	+	>200	–
—CH=N—O—CH$_2$C$_6$H$_5$	0·005	+	20	+
—CH=N—O—C$_5$H$_{11}$	0·01	+	10	+
—CH=N—O—C$_8$H$_{17}$	0·1	+	1·0	+
—N⟨ ⟩	0·04	+	4–8	–
—N⟨ ⟩CH(CH$_3$)$_2$	0·009	+	1–2	–

emergence of organisms insensitive to them. As mentioned, in the resistant mutants the subunit β of the RNA polymerase is modified and is no more affected by rifampicin and by the other rifamycins in clinical use.

Testing hundreds of semisynthetic rifamycins, it has been found that some groups of derivatives inhibit, at concentration inferior to 20 μg ml^{-1}, the growth of a Staphylococcus strain resistant to more than 200 μg ml^{-1} of rifampicin. In *Table 6* two groups of derivatives are reported: the oximes of the 3-formylrifamycin SV[73], and the 3-*N*,*N*-disubstituted aminorifamycins[74].

Although the oximes do have a certain activity on the isolated RNA polymerase from rifampicin-resistant bacteria, it cannot be concluded that this is the only responsible mechanism of inhibition of the intact bacteria. The oximes inhibit other enzymes as well (see reverse transcriptase p. 407) and have lost, at least partially, the specificity of the parent molecule.

The 3-*N*,*N*-disubstituted aminorifamycins active on *S. aureus* resistant to rifampicin had no effect on the RNA polymerase extracted from the resistant cells. In this case a different mechanism of action should be responsible for this activity on resistant mutants. The compounds reported in *Table 6* have no practical interest, because their minimal inhibitory concentration is too high to foresee a therapeutic use for them, but the case of oximes is indicative that some structural modifications of the rifamycin molecule could permit the obtaining of inhibitors of the RNA polymerase resistant to rifampicin.

ACTIVITY OF RIFAMYCINS ON VIRAL TRANSCRIPTASES

Transcriptase from mammalian cytoplasmic DNA viruses

A DNA-dependent RNA polymerase is contained in the virions of some large mammalian cytoplasmic DNA viruses such as pox viruses[75]. Specific inhibitors of this enzyme might be potentially useful antiviral agents. Rifampicin was found to inhibit the growth of pox viruses, but its viral inhibitory dose (100 μg ml^{-1}) is from 1000 to 10 000 times higher than the antibacterial one[76,77]. The mechanism of the antiviral activity of rifampicin has been the object of many studies with conflicting conclusions. Although the antibiotic shows some action on the transcription of the viral genome, the inhibition of vaccinia virus growth seems to be related to a block in the assembly of preformed structural polypeptides of the virion[78–80]. Other evidence that the mechanism of action of rifampicin against pox viruses is different from that against bacteria derives from the fact that virion-associated RNA polymerase of resistant mutants is sensitive to the same concentrations of rifampicin as the wild-type[81]. Furthermore, a number of different rifamycin derivatives active on the bacterial polymerase are not inhibitory of vaccinia plaque formation[81,82]. On the other hand, it has been reported that one rifamycin derivative, 23-dehydroxy-27-demethoxy-23,27 epoxyrifamycin SV (see *Figure 10*), which does not react with the bacterial polymerase, inhibits the vaccinia virus enzyme[83]. This fact indicates that some structural changes on the ansa chain could confer on the rifamycin molecule the property of blocking the viral enzyme. Assuming that the mechanism of action of rifampicin is different in bacteria and viruses, the discovery of its antiviral activity can be considered as a case of serendipity.

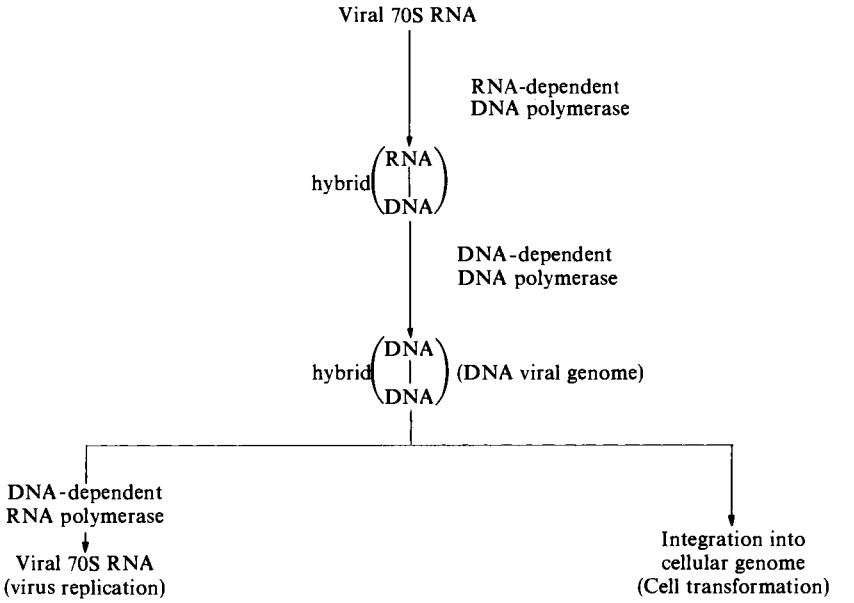
Until now neither rifampicin nor other semisynthetic rifamycins show significant therapeutic activity in experimental viral infections.

Reverse transcriptase (RNA-dependent DNA polymerase) from oncogenic RNA viruses

It is known that tumours can be induced in animals by various chemical, physical and biological triggers. Many DNA and RNA viruses are oncogenic in animals. They do not multiply in the cell causing its death, but determine a morphological transformation of the cell, whose subsequent multiplication is in part controlled by viral genes, integrated into the cell genome. In the case of DNA tumour viruses, replication of the viral genetic material and its expression is basically in symbiosis with the host metabolism. Also for the RNA oncogenic viruses, which constitute a large group of animal viruses, also called leukoviruses, the viral information is integrated into the cell genome and is transmitted to the daughter cells.

The structure containing the viral information was called provirus by Temin[84], who hypothesized in 1964 that replication of RNA tumour viruses had to involve a DNA intermediate using the viral RNA as template. This hypothesis was at that time in contrast with the established concept of information transfer, but was confirmed in 1970 when Temin and Mizutani[85] and Baltimore[86] independently demonstrated the presence of the RNA-dependent DNA polymerase in Rous sarcoma virus and in Rauscher leukaemia virus. The enzyme is called also 'reverse transcriptase' and its presence has been confirmed in at least 40 RNA oncogenic viruses. The non-oncogenic RNA viruses do not contain an RNA-dependent DNA polymerase activity.

The RNA tumour virus replication and cell transformation can be indicated schematically as shown below:



RNA-dependent DNA polymerase activity has been found in the milk of women from families with a history of breast cancer. Such milk was also found to contain particles morphologically identical to the type B mouse mammary tumour virus[87]. A similar enzymatic activity has been identified in leukocytes of some patients with acute leukaemia and not in lymphocytes of healthy subjects even when mitosis was induced with phytohaemagglutinin[88,89]. The molecular weight of the enzymes from avian myeloblastosis virus and from Rous sarcoma virus have been reported to be about 110 000 and that from Rauscher leukaemia virus about 70 000[90–92]. The structure of the enzyme(s) is not known.

The RNA-dependent DNA polymerase activity is sensitive to ribonuclease and requires all four deoxyribonucleotides for the reaction. The activity is stimulated by the addition of exogenous, synthetic DNA–RNA hybrid templates.

The reverse transcriptase of RNA tumour viruses of human acute leukaemic cells can be distinguished from known normal cellular DNA-directed DNA polymerases on the basis of its response to certain oligomer–homopolymer complexes[93,94]. In fact, reverse transcriptase responds very well to oligo(dT).poly(rA) templates and very poorly to oligo(dT).poly(dA) templates, while DNA polymerases of calf thymus or normal human lymphocytes and of bacteria have a different behaviour. The ratio of DNA polymerase activity using the two indicated oligopolymer–homopolymer complexes as templates is indicative of the reverse transcriptase activity.

Although the aetiology of human neoplasia is not known, a number of hypotheses implicate the reverse transcriptase at some stage in the process of cancerogenesis.

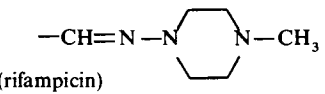
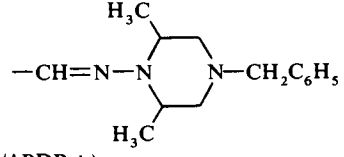
Inhibitors of the reverse transcriptase could constitute a powerful tool for understanding the role of reverse transcriptase in viral cancerogenesis, and perhaps could have an inhibitory effect on tumour induction or on tumour growth. Rifamycins and streptovaricins have been tested for their effect on reverse transcriptase.

Rifampicin was found to be inactive, but some derivatives with modified aminopiperazine side chains showed an inhibitory effect on reverse transcriptase of MSV (murine sarcoma virus) Fe LV (feline leukaemia virus) and AMV (avian myeloblastosis virus). The most active ones were initially indicated to be rifamycins AF/ABDP (2,6-dimethyl-4-benzyl-4-demethyl-rifampicin), AF/ABP (4-benzyl-4-demethyl-rifampicin) and AF/AP (4-demethyl-rifampicin)[95,96]. The three rifamycins inhibited the enzyme activity more than 50 per cent at 50 to 100 $\mu g\ ml^{-1}$. The most effective inhibitor, AF/ABDP, blocked 95 per cent to 100 per cent of the enzyme activity at 100 $\mu g\ ml^{-1}$. 4-Demethylrifampicin caused complete inhibition only at a concentration $>200\ \mu g\ ml^{-1}$.

The streptovaricin complex has been reported to cause a 75 per cent inhibition of the reverse transcriptase activity of MLV (Moloney leukaemia virus) at a concentration of 40 $\mu g\ ml^{-1}$[97].

Other *in vitro* effects of ansamycins on the oncogenic RNA viruses have been observed. Rifampicin inhibits focus formation in chick embryo cells infected by Rous sarcoma virus[98] and rifamycin AF/ABDP inhibits transformation of mouse cells by murine sarcoma virus[99].

Table 7. Selected biological data on some rifamycins active and inactive on the reverse transcriptase

R	Inhibitory effect on RLV reverse transcriptase at 100 μg ml^{-1} [104] (%)	Minimal inhibitory concentration on [73]			Inhibitory effect on DNA-dependent RNA polymerase A and B from calf thymus [103]								I.V. acute toxicity in mice (ED_{50} mg kg^{-1})
		S. aureus (μg ml^{-1})	*S. aureus* +20% B.S.A. (μg ml^{-1})	Rifampicin resistant *S. aureus* (μg ml^{-1})	A (μg ml^{-1})				B (μg ml^{-1})				
					20	40	100	400	20	40	100	400	
 (rifampicin)	0	0·002	0·005	>200	—	0	—	3	0	—	—	3	330
 (AF/ABDP*cis*)	72	0.001	0.5	20	20	70	95	100	16	74	100	100	61
—CH=N—O—C_8H_{17} (AF/013)	75	0.1	5	1	93	—	—	100	100	—	100	—	88

All these data stimulated a search for potent inhibitors of the reverse transcriptase and more than 200 rifamycin derivatives were tested for their effect on this enzyme[100,101]. Whereas the majority of semi-synthetic rifamycins are inactive, or moderately active, some derivatives are quite effective inhibitors, blocking the RNA-dependent DNA polymerase reaction at concentrations of less than 20 μg ml^{-1}. Most of these compounds have bulky substituents in position 3, e.g. the hydrazones and oximes of 3-formyl rifamycin SV. Also the 3-cyclic amino derivatives of rifamycin SV that contain cyclohexyl substituents, exhibit a high degree of activity on DNA polymerase of MSV[102]. This extensive screening has demonstrated the possibility of reaching a relatively high activity against reverse transcriptase through adequate modification of the rifamycin molecule. But a question immediately arises concerning the selectivity of action of these derivatives. In a screening of certain rifamycin derivatives for their effect on animal polymerases it was found that some of them completely inhibit the calf thymus DNA-dependent RNA polymerase AI and B activities at concentrations of 20 to 40 μg ml^{-1} [103].

Among the most active are the same derivatives, such as rifamycin AF/ABDP and rifamycin AF/013 (octyloxime of 3-formyl rifamycin SV), selected for their high activity on reverse transcriptase. The lack of specificity of some of these derivatives is indicated by some selected biological data reported in *Table 7*[103,104]. The two derivatives AF/ABDP *cis* and AF/013, active on RLV reverse transcriptase, show a remarkable loss of activity against sensitive *S. aureus* strain in the presence of bovine serum albumin, good activity against a *S. aureus* strain resistant to rifampicin, high inhibitory effect on animal DNA-dependent RNA polymerases and a remarkably acute toxicity in mice. Therefore it seems that these derivatives cannot be used as specific inhibitors of RNA-dependent DNA polymerase.

On the contrary rifampicin, which is inactive on reverse transcriptase and selectively active on bacterial RNA polymerase, shows only a minor loss of antibacterial activity in the presence of bovine serum albumen, no activity against animal RNA polymerases and is less toxic than the other compounds.

All these data tend to indicate that for some rifamycin derivatives a higher activity against reverse transcriptase was achieved together with a broader spectrum of activity against other transcriptases and therefore with poor selectivity. This could not be the case for other derivatives and in fact Gallo *et al.*[104] pointed out that *N*-demethylrifampicin and 3-(2,4-dinitrophenylhydrazonomethyl) rifamycin SV inhibit leukaemic polymerases more strongly than the analogous normal enzymes (*Table 8*). Furthermore some rifamycin derivatives, active on reverse transcriptases of both human and viral origin, have been found to be more toxic for fresh human leukaemic blood cells than for normal proliferating blood cells[105]. Although the mechanism of the selective toxicity for the leukaemic blood cells is not clarified, and is not necessarily related to the inhibitory activity on reverse transcriptase, this effect could be chosen for the selection of potentially useful chemotherapeutic agents.

In summary, the screening of many semisynthetic rifamycins for their activity on reverse transcriptase revealed a series of potent, but not specific, inhibitors of this polymerase. Only a few derivatives seem to have a moderate specificity of action against this enzyme. On the other hand, viral and cellular polymerases have remarkable structural differences as indicated by their

Table 8. Relative differences between inhibition of purified DNA polymerase activities from leukaemic and normal (1788) lymphoblasts[104]

Rifamycin	Inhibitory concentration for 50% inhibition ($\mu g\ ml^{-1}$) (Poly d(AT) template)			
	DNA polymerase I		DNA polymerase II	
	1788	Leukaemic	1788	Leukaemic
R = —CH=N—N NH (*N*-demethylrifampicin)	>1000	>1000	>750	100
R = —CH=N—NH— O_2N, NO_2	16	5	10	<6

different template specificities and cellular functions[102]. Therefore, at least theoretically, it should be possible to develop specific inhibitors of the DNA polymerase of RNA tumour viruses.

A systematic study of semisynthetic or new natural ansamycins with new modifications both in the aromatic nucleus and on the ansa chain could perhaps lead to a knowledge of the structural requirements for the specific inhibitory effect on reverse transcriptase. A product with such a property will be a powerful tool for understanding the role of the reverse transcriptase in the process of tumour induction and propagation. Furthermore, if the enzyme plays a definite role in these processes, a specific inhibitor will have the necessary requisite of a potential chemotherapeutic agent.

ACKNOWLEDGMENT

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REFERENCES

[1] R. Ferone, J. J. Burchall and G. H. Hitchings, *Mol. Pharmacol.* **5**, 49 (1969).
[2] S. P. Biatti, C. J. Ingles, L. J. Lindell, P. W. Morris, R. F. Weaven, F. Weinberg and W. J. Rutter, *Cold Spring Harbor Symp. Quant. Biol.* **35**, 649 (1970).
[3] R. F. Weaven, S. P. Biatti and W. J. Rutter, *Proc. Nat. Acad. Sci. US*, **68**, 2994 (1971).
[4] R. R. Burgess, A. A. Travers, J. J. Dunn and E. K. F. Bautz, *Nature*, **221**, 43 (1969).
[5] T. Wieland, *Science*, **159**, 946 (1968).
[6] L. Fiume and F. Stirpe, *Biochim. Biophys. Acta*, **123**, 643 (1966).
[7] T. J. Lindell, F. Weinberg, P. W. Morris, R. G. Roeder and W. J. Rutter, *Science*, **170**, 447 (1970).
[8] S. Dezelee, A. Sentenac and P. Fromageot, *FEBS Lett.* 7, 220 (1970).

[9] L. K. Rinehart, J. R. Beck, D. B. Borders, T. H. Kinstle and D. Krauss, *J. Am. Chem. Soc.* **85**, 4038 (1963).
[10] M. Gniadowski, J. L. Mandel, F. Gissinger, C. Kedinger and P. Chambon, *Biochem. Biophys. Res. Commun.* **38**, 1033 (1970).
[11] G. Cassani, R. R. Burgess, A. M. Goodman and L. Gold, *Nature New Biology*, **230**, 197 (1971).
[12] P. Sensi, P. Margalith and M. T. Timbal, *Farmaco (Pavia) Ed. Sci.* **14**, 146 (1959).
[13] P. Sensi, A. M. Greco and R. Ballotta, *Antibiotics Ann.* 262 (1959).
[14] V. Prelog, *in The chemistry and biochemistry of fungi and yeasts* 551, Butterworths, London (1963).
[15] W. Oppolzer, V. Prelog and P. Sensi, *Experientia*, **20**, 336 (1964).
[16] J. Leitich, W. Oppolzer and V. Prelog, *Experientia*, **20**, 343 (1964).
[17] M. Brufani, W. Fedeli, G. Giacomello and A. Vaciago, *Experientia*, **20**, 339 (1964).
[18] J. Leitich, V. Prelog and P. Sensi, *Experientia*, **23**, 505 (1967).
[19] M. Brufani, W. Fedeli, G. Giacomello and A. Vaciago, *Experientia*, **23**, 508 (1967).
[20] G. C. Lancini, G. G. Gallo, G. Sartori and P. Sensi, *J. Antibiotics*, **22**, 369 (1969).
[21] G. C. Lancini, C. Hengeller and P. Sensi, *in Progress in Antimicrobial and Anticancer Chemotherapy* Part II, 1166, Univ. Tokyo Press, Tokyo (1970).
[22] S. Sugawara, K. Karasawa, M. Watanabe and T. Hidaka, *J. Antibiotics*, **17**, 29 (1964).
[23] I. Takagi, S. Iriyama and S. Umezawa, *Proc. Fujihara Mem. Fac. Eng. Keio Univ. (Tokyo)*, **19**, 163 (1966).
[24] G. C. Lancini and C. Hengeller, *J. Antibiotics*, **22**, 637 (1969).
[25] T. Kishi, M. Asai, M. Muroi, S. Harada, E. Mizuta, S. Teroo, T. Miki and K. Mizuno, *Tetrahedron Letters*, 91 (1969).
[26] T. Kishi, S. Harada, M. Asai, M. Muroi and K. Mizuno, *Tetrahedron Letters*, 97 (1969).
[27] P. Siminoff, R. M. Smith, W. T. Sokolski and G. M. Savage, *Am. Rev. Resp. Diseases*, **75**, 576 (1957).
[28] K. L. Rinehart, M. L. Maheshwari, K. Sasaki, A. J. Schacht, H. H. Mathur and F. J. Antosz, *J. Am. Chem. Soc.* **93**, 6273 (1971).
[29] K. L. Rinehart and J. F. Antosz, *J. Antibiotics*, **25**, 71 (1972).
[30] C. Calvori, L. Frontali, L. Leoni and G. Tecce, *Nature*, **207**, 417 (1965).
[31] H. Umezawa, S. Mizuno, H. Yamazaki and K. Nitta, *J. Antibiotics*, **21**, 234 (1968).
[32] G. Hartmann, K. O. Honikel, F. Knusel and J. Nuesch, *Biochem. Biophys. Acta*, **145**, 843 (1967).
[33] G. C. Lancini and G. Sartori, *Experientia*, **24**, 1105 (1968).
[34] G. C. Lancini, R. Pallanza and L. Silvestri, *J. Bacteriol.* **97**, 761 (1969).
[35] E. Winterzberger and V. Winterzberger, *FEBS Letters*, **6**, 58 (1970).
[36] S. Yang and R. S. Criddle, *Biochemistry*, **9**, 3063 (1970).
[37] F. Herzfeld, *Z. Physiol. Chem.* **351**, 658 (1970).
[38] D. T. Dubin and B. S. Montenecourt, *J. Mol. Biol.* **48**, 279 (1970).
[39] M. N. Gadaleta, M. Greco and C. Saccone, *FEBS Letters*, **10**, 54 (1970).
[40] J. G. Gamble and R. H. Mc. Cluer, *J. Mol. Biol.* **54**, 557 (1970).
[41] Z. G. Schmerling, *Biochem. Biophys. Res. Commun.* **37**, 965 (1969).
[42] A. Sippel and G. Hartmann, *Biochem. Biophys. Acta*, **157**, 218 (1968).
[43] H. Lill, U. Lill, A. Sippel and G. Hartmann, *in RNA Polymerase and transcription* 55. Proc. 1st Lepetit Colloq. North-Holland Publishing Co., Amsterdam (1970).
[44] A. Sippel and G. Hartmann, *Eur. J. Biochemistry*, **16**, 152 (1970).
[45] V. Neuhoff, W. B. Schill and H. Sternbach, *Z. Physiol. Chem.* **350**, 335 (1969).
[46] W. Wehrli, K. Knüsel, K. Schmid and M. Staehelin, *Proc. Nat. Acad. Sci. US*, **61**, 667 (1968).
[47] W. Wehrli and M. Staehelin, *Biochim. Biophys. Acta*, **182**, 24 (1969).
[48] W. Wehrli and M. Staehelin, *in RNA Polymerase and transcription* 65, Proc. 1st Int. Lepetit Colloq. North-Holland Publishing Co., Amsterdam (1970).
[49] W. Wehrli, F. Knusel and M. Staehelin, *Biochem. Biophys. Res. Commun.* **32**, 284 (1968).
[50] G. P. Tocchini-Valentini, P. Marino and A. J. Calvill, *Nature*, **220**, 275 (1968).
[51] A. Heil and W. Zillig, *FEBS Letters*, **11**, 165 (1970).
[52] W. Zillig, E. Fuchs, P. Palm, D. Rabussay and K. Zechel, *in RNA Polymerase and transcription* 151. Proc. 1st Int. Lepetit Colloq. North-Holland Publishing Co., Amsterdam (1970).
[53] D. Rabussay and W. Zillig, *FEBS Letters*, **5**, 104 (1969).
[54] N. Maggi, S. Furesz and P. Sensi, *J. Med. Chemistry*, **11**, 368 (1968).
[55] P. Sensi, R. Ballotta, A. M. Greco and G. G. Gallo, *Farmaco (Pavia) Ed. Sci.* **16**, 165 (1961).
[56] P. Sensi, N. Maggi, S. Furesz and Maffii. *Antimicrobial Agents and Chemotherapy*, 699 (1966).
[57] N. Maggi, A. Vigevani, G. G. Gallo and C. R. Pasqualucci, *J. Med. Chem.* **11**, 936 (1968).

[58] N. Maggi, A. Vigevani and R. Pallanza, *Experientia*, **24**, 209 (1968).
[59] H. Bickel, F. Knusel, W. Kump and L. Neipp, *Antimicrobial Agents and Chemotherapy*, 352 (1966).
[60] R. J. White—Personal communication.
[61] P. Sensi, N. Maggi, R. Ballotta, S. Furesz, R. Pallanza and V. Arioli, *J. Med. Chem.* 7, 596 (1964).
[62] A. M. Greco, R. Ballotta and P. Sensi, *Farmaco (Pavia) Ed. Sci.* **16**, 755 (1961).
[63] P. Sensi, M. T. Timbal and A. M. Greco, *Antibiot. Chemotherapy*, **12**, 488 (1962).
[64] R. Cricchio and G. Tamborini, *J. Med. Chem.* **14**, 721 (1971).
[65] G. G. Gallo, C. R. Pasqualucci, N. Maggi, R. Ballotta and P. Sensi, *Farmaco (Pavia) Ed. Sci.* **21**, 68 (1966).
[66] N. Maggi, V. Arioli and G. Tamborini, *Farmaco (Pavia) Ed. Sci.* **24**, 263 (1969).
[67] N. Maggi and R. Pallanza, *Farmaco (Pavia) Ed. Pr.* **22**, 307 (1967).
[68] N. Maggi, V. Arioli and P. Sensi, *J. Med. Chem.* **8**, 790 (1965).
[69] N. Maggi, R. Pallanza and P. Sensi, *Antimicrobial Agents and Chemotherapy*, 765 (1965).
[70] S. Furesz, V. Arioli and R. Pallanza, *Antimicrobial Agents and Chemotherapy*, 770 (1965).
[71] N. Maggi, C. R. Pasqualucci, R. Ballotta and P. Sensi, *Chemotherapy*, **11**, 285 (1966).
[72] G. Binda, E. Domenichini, A. Gottardi, B. Orlandi, E. Ortelli, B. Pacini and G. Fowst, *Arzneim. Forsch. (Drug Res.)*, **21**, 1907 (1971).
[73] Unpublished results.
[74] F. Knusel, H. Bickel and W. Kump, *Experientia*, **25**, 1207 (1969).
[75] J. R. Kates and B. R. McAuslan, *Proc. Nat. Acad. Sci. US*, **58**, 134 (1967).
[76] E. Heller, M. Argamon, H. Levy and N. Goldblum, *Nature*, **222**, 273 (1969).
[77] J. H. Subak-Sharpe, M. C. Timbury and J. F. Williams, *Nature*, **222**, 341 (1969).
[78] T. H. Pennington, E. A. Follett and J. F. Szilagyi, *J. Gen. Virol.* **9**, 225 (1970).
[79] B. Moss, E. N. Rosenblum and P. M. Grimley, *Virology*, **45**, 123 (1971).
[80] B. Moss, E. N. Rosenblum and P. M. Grimley, *Virology*, **45**, 135 (1971).
[81] J. H. Subak-Sharpe, T. H. Pennington, J. F. Szilagyi, M. C. Timbury and J. F. Williams, *in RNA Polymerase and Transcription* 260, Proc. 1st Lepetit Colloq. North-Holland Publishing Co., Amsterdam (1970).
[82] Z. Zakay and Y. Becker, *Nature*, **226**, 1162 (1970).
[83] J. F. Szilagyi and T. H. Pennington, *J. Virol.* **8**, 133 (1971).
[84] H. M. Temin, *Virology*, **23**, 486 (1964).
[85] H. M. Temin and S. Mizutani, *Nature*, **226**, 1211 (1970).
[86] D. Baltimore. *Nature*, **226**, 1209 (1970).
[87] J. Schlom, S. Spiegelman and D. Moore, *Nature*, **231**, 97 (1971).
[88] R. C. Gallo, S. S. Yang and R. C. Ting, *Nature*, **228**, 927 (1970).
[89] R. C. Gallo, S. S. Yang, R. G. Smith, F. Herrera, R. C. Ting, S. N. Bobrow, C. Davis and S. Fujoka, *in The Biology of Oncogenic Viruses* 210, Proc. 2nd Lepetit Collq. North-Holland Publ. Co., Amsterdam (1971).
[90] D. L. Kacian, R. F. Watson, A. Burny and S. Spiegelman, *Biochim. Biophys. Acta*, **246**, 365 (1971).
[91] P. Duesberg, K. V. D. Helm and E. Canaani, *Proc. Nat. Acad. Sci. US*, **68**, 2505 (1971).
[92] D. Baltimore and D. Smiler, *Proc. Nat. Acad. Sci. US*, **68**, 1507 (1971).
[93] N. C. Goodman and S. Spiegelman, *Proc. Nat. Acad. Sci. US*, **68**, 2203 (1971).
[94] M. S. Robert, R. G. Smith and R. C. Gallo, *Science*, **176**, 798 (1972).
[95] M. Green, M. Rokutanda, K. Fujinaga, H. Rokutanda, C. Gurgo, R. K. Ray and J. T. Parsons, *in The Biology of Oncogenic Viruses*, 193. Proc. 2nd. Lepetit Colloq. North-Holland Publ. Co., Amsterdam (1971).
[96] C. Gurgo, R. K. Ray, L. Thiry and M. Green, *Nature*, **229**, 111 (1971).
[97] W. W. Brockman, W. A. Carter, L. Li, F. Reusser and L. R. Nichol, *Nature*, **230**, 249 (1971).
[98] H. Diggelman and C. Weissman, *Nature*, **224**, 1277 (1969).
[99] M. Calvin, U. R. Joss, A. J. Hackett and R. B. Owens, *Proc. Nat. Acad. Sci. US*, **68**, 1441 (1971).
[100] R. S. Yang, F. Herrera, R. G. Smith, M. Reitz, G. C. Lancini, R. C. Ting and R. C. Gallo, *J. Nat. Cancer Inst.* (in press).
[101] C. Gurgo, R. Ray and M. Green, *J. Nat. Cancer Inst.* (in press).
[102] M. Green, *Proc. Nat. Acad. Sci. US*, **69**, 1036 (1972).
[103] M. Meilhac, Z. Tysper and P. Chambon, *Eur. J. Biochem.* **28**, 291 (1972).
[104] R. C. Ting, S. S. Yang and R. C. Gallo, *Nature New Biology*, **236**, 163 (1972).
[105] R. G. Smith, J. Whang-Peng, R. C. Gallo, P. Levine, T. C. Ting, *Nature New Biology*, **236**, 166 (1972).

ANALOGUES OF CYCLIC AMP AND THEIR PHYSIOLOGICAL RESPONSE

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ABSTRACT

Sutherland's second messenger model and the intracellular cAMP†-system is presented. The key-points of its manipulation by externally applied compounds are discussed. They form the guidelines for development of chemical analogues of the cAMP molecule effective on phosphodiesterases and protein kinases.

Special attention is devoted to their influence on glycogenolysis, steroidogenesis, lipolysis, hormone secretion and contractility of various muscles. Some of these analogues show relatively high specificity of physiological responses, such as separation of metabolic and contractile effects, while others show general enhancement of the multivalent responses of cAMP itself. It is shown that a good correlation between *in vitro* and *in vivo* data exist. The pharmacological significance of these findings is briefly discussed.

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† Abbreviations:

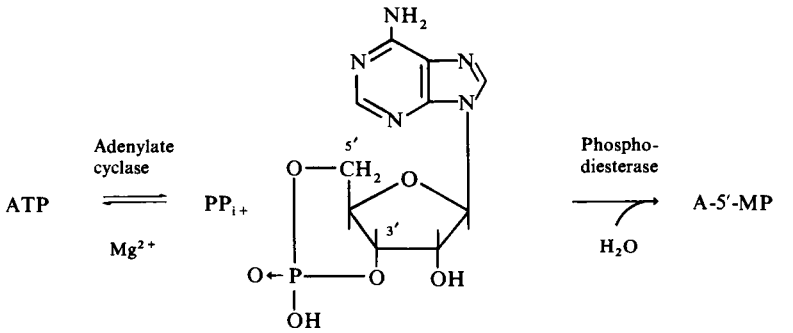
AICAR: 4-amino-imidazole-5-carboxamide riboside
ACTH: adrenocorticotropic hormone
A-5'-MP: adenosine-5'-monophosphate
ASN: adenosine
ATP: adenosine triphosphate
cAMP: adenosine-3':5'-monophosphate, cyclic AMP
cCMP: cytidine-3':5'-monophosphate, cyclic CMP
cdAMP: desoxyadenosine-3':5'-monophosphate, cyclic dAMP
cdTMP: desoxythymidine-3':5'-monophosphate, cyclic dTMP
cGMP: guanosine-3':5'-monophosphate, cyclic GMP
cIMP: inosine-3':5'-monophosphate, cyclic IMP
cTuMP: tubercidine-3':5'-monophosphate, cyclic TuMP
cUMP: uridine-3':5'-monophosphate, cyclic UMP
cXMP: xanthosine-3':5'-monophosphate, cyclic XMP
DBcAMP: N^6,2'-O-dibutyryl-adenosine-3':5'-monophosphate
DBcGMP: N^2,2'-O-dibutyryl-guanosine-3':5'-monophosphate
DG: digylceride
FFA: free fatty acids
GTP: guanosine triphosphate
MG: monoglyceride
PDE: 3':5'-phosphodiesterase
PIA: N^6-phenylisopropyl-adenosine
PP_i: pyrophosphate
RH: releasing hormone
TG: triglyceride
TSH: thyreostimulating hormone

THE SECOND MESSENGER MODEL

The humoral regulation of various tissue functions in mammals depends on information transmitters, usually called 'hormones'. They are generated in secretory tissues, from which they are released as a response to neural or humoral stimuli. Thus, they transfer the information to the target organ. As an example, the regulation process in the thyroid system functions as follows: humoral and neural signals stimulate a primary endocrine gland (hypothalamus), the secreted hormone (TSH releasing factor) reaches via the circulation a dependent gland (pituitary gland), where the process is repeated. The secreted hormone (TSH) finally reaches the target organ (thyroid gland), exerts its influence on the tissue and is subsequently metabolized. This decrease in hormone concentration, in turn, may be a stimulus for renewed action of the primary endocrine gland.

Sutherland and his coworkers[1]† were the first to show that an intracellular increase of an adenosine nucleotide with a cyclic phosphate ester group (cAMP) precedes the hormone regulated events in the target tissues.

Formation and degradation of cAMP is regulated by two ubiquitous enzymes (*Figure 1*), the ATP cyclase and the 3′:5′-phosphodiesterase (PDE). ATP cyclase forms an intramolecular ester-bond between the α-phosphorus of ATP and the 3′-hydroxyl group, releasing inorganic pyrophosphate. The equilibrium of the reaction does not favour cAMP formation. In some microorganisms the ATP cyclase is found in the cytoplasm, while the enzyme in cariontes occurs in membrane-bound form. When the enzyme is dissociated, for example by adding detergents, its capacity for activation by hormones is lost or drastically reduced.



Adenosine-3′:5′-monophosphate
(cAMP)

Figure 1. Formation and degradation of cAMP.

The intracellular cAMP-levels are strongly influenced by specific phosphodiesterases, which cleave the 3′:5′-cyclophosphate ring, forming the corresponding nucleoside-5′-monophosphate. The enzyme, isolated from various sources, is soluble and localized to a great part in the cytoplasm.

† For comprehensive literature see the monograph of Sutherland *et al.*[2]

Since the discovery of Sutherland most hormones have been found to activate the ATP cyclase and thus elevate the intracellular cAMP-level (*Table 1*). The hormone-regulated ATP cyclase is the centrepiece of Sutherland's second messenger concept (review by Robison *et al.*[2]; *Figure 2*) and consequently, is the key to our current understanding of hormone action. The particle-bound cyclase seems to be connected with a series of receptors, that differentiate between the various arriving hormones. One can assume, that the cell-surfaces of different tissues show different receptor patterns, and, therefore, they are stimulated by different hormones. Our present knowledge of the molecular mechanism of this hormone-induced activation

Table 1. Some hormones which influence the intracellular concentration of cyclic AMP

Prostaglandins	Adrenocorticotropic hormone
Catecholamines	Thyroid releasing factor
Glucagon	Thyroid stimulating hormone
Vasopressin	Melanocyte stimulating hormone
Histamine	Parathyroid hormone
Serotonin	Luteotropic hormone

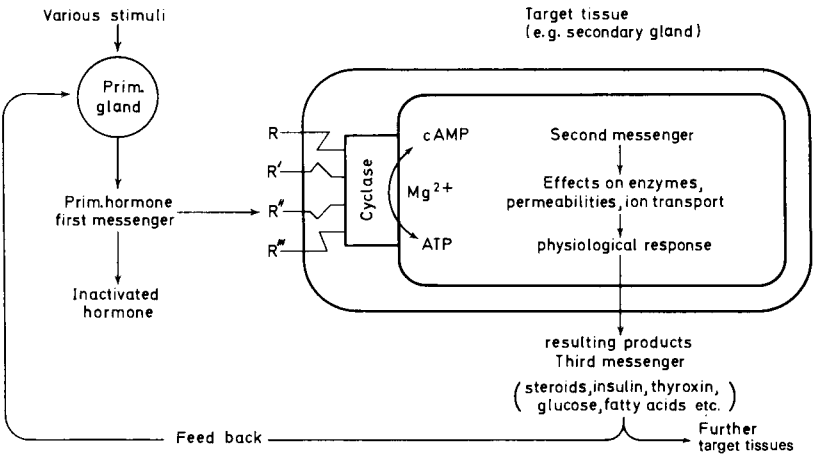


Figure 2. Second messenger model (modified according to Sutherland). The primary response to a hormonal signal is the intracellular formation of cAMP which, in turn, produces typical effects. Compounds formed this way may act as hormonal signals in the extracellular space again.

process is limited. Possibly prostaglandins are involved. The products resulting from the cellular response of the cAMP may influence both the secretory gland via a feedback mechanism, and a further target tissue, possibly by activating a cyclase again. In this chain of events the hormone is the first messenger and the cAMP the second.

It is surprising how many effects arise from the single event, cAMP elevation, such as changes in enzyme activity, in membrane potentials and in ion flux, until the effects of cAMP are neutralized by the specific PDE. A partial list of these actions is shown in *Table 2*. In liver, glycogenolysis, gluconeogenesis, urea formation and inhibition of lipid synthesis occurs, while in adipose tissue, lipolysis is stimulated. Generally speaking, in these tissues, stimulation of catabolic pathways prevails. The effects on contractile tissues as well as on hormone release will be a further subject of this paper.

In contrast to ATP, which represents the universal energy transmitter of the living cell, cAMP is, most likely, the universal intracellular information transmitter. The cAMP-concentration within the cell is about 10^{-7}M and is drastically lower than the concentration of other adenine nucleotides (e.g. ATP, ca. 10^{-3}M). cAMP apparently can cross the cell wall barrier from the intracellular to the extracellular space (but not in the reverse direction!), since cAMP is found in blood and in urine.

Table 2. Effects of cyclic AMP in several organs

	Metabolic effects	
Liver	Glycogenolysis	increased
	Gluconeogenesis	increased
	Urea formation	increased
	Ketogenesis	increased
	Amino acid uptake	increased
	Amino acid → protein	decreased
	Lipogenesis	decreased
	Net K^+- and Ca^{2+}-efflux	increased
Adipose tissue	Lipolysis	increased
	O_2-consumption	increased
Kidney	Permeability (Tubules)	increased
	Renin production	increased
Bone	Ca^{2+}resorption	increased
Cardiac muscle	Ca^{2+} uptake	increased
Gastric mucosa	HCl secretion	increased
	Other tissue effects	
Smooth muscle	Relaxation	increased
Cardiac muscle	Rate of contraction	increased
	Force of contraction	increased
Platelets	Aggregation	decreased
Toad bladder	Permeability	increased
Cerebellar Purkinje cells	Discharge frequency	decreased
	Hormone release	
Anterior pituitary	Adrenocorticotropic hormone	increased
	Thyroid stimulating hormone	increased
	Growth hormone	increased
Thyroid	Thyroid hormone	increased
	Calcitonin	increased
Pancreas		
Exocrine	Amylase	increased
Islets	Insulin	increased
Adrenal (Cortex)	Corticosteroids	increased

THE cAMP-SYSTEM

One is tempted to postulate that the multiplicity of biochemical events caused by the presence of cAMP is based on a common principle of action. Indeed, the discovery of cAMP-sensitive protein kinases in various tissues supports this assumption[3,4]. Protein kinases phosphorylate other catalytic proteins and thus modulate their activity. In general, protein kinases consist of regulatory and catalytic subunits. Binding of cAMP to the regulatory site causes the complex to dissociate, thereby releasing the enzymatically active, catalytic subunit of the protein kinase. The physiological substrates of the various protein kinases are not yet completely known. Their identification is necessary in order to clarify the mechanism of action of the second messenger in the different tissues.

Besides regulation of vital cell processes by cAMP, there is good evidence that cGMP may play an important role, too (for a review, see Hardman *et al.*[5]). The tissue level of cGMP is lower than that of cAMP by a factor of 10 to 100. The GTP cyclase which forms cGMP from GTP is partially soluble unlike the ATP cyclase. cGMP catabolism seems to be controlled by G-specific phosphodiesterases, though each nucleotide inhibits the other's hydrolysis. cGMP appears to stimulate other protein kinases than cAMP. These facts imply that the spectrum of its biochemical effects is shifted, as compared to cAMP. The significance of the physiological occurrence of cGMP has not been ascertained so far.

The same holds true for various protein factors that seem to control the activity of phosphodiesterases and protein kinases[6–8].

These more or less well established facts are compiled in *Figure 3*. The chain of events is started by humoral or neural input signals. By elevation of the cAMP level, enzyme activities are modulated either by direct effects or

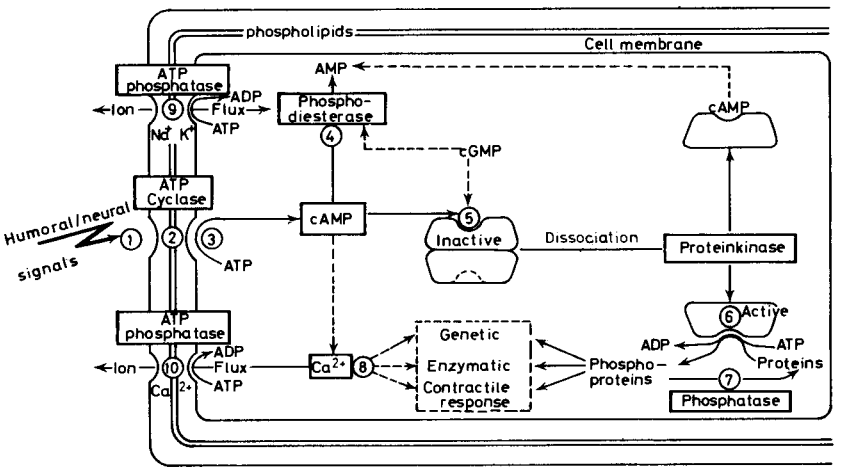


Figure 3. Keypoints of attack in the cAMP-system. The mechanism of the events from the hormonal input signal to the cellular response is shown. Solid lines indicate well established reactions, dashed lines hypothetical ones. Figures indicate points where the cAMP system may be influenced. For details see the text.

via triggering the protein synthesis. These processes alter the dynamic situation of various metabolic pathways. Other tissue effects such as relaxation of the smooth muscle or increase of the force of contraction in the cardiac muscle are controlled by cAMP, at least partly by the regulation of the ion-transport, especially of the calcium flux. Both principles may be involved in the stimulation of hormone secretion by the second messenger.

cAMP-effects depend primarily on the activities of ATP-cyclase, phosphodiesterase and protein kinase. Key positions for the modulations of the second messenger system are numbered in *Figure 3*. They are the ATP cyclase, consisting of the receptor site where the hormone signal is received (1), the phospholipid layer (2), and the catalytic site at the inner side of the plasma membrane (3), and further the phosphodiesterase (4), the regulatory site of the protein kinase (5) and subsequently its catalytic centre (6) after activation has taken place. In addition, indirect effects on the system may play an important role, e.g. phosphatases can deactivate phosphoproteins by dephosphorylation. Our present knowledge about the influence of cAMP-level on ion transport, especially on the calcium flux, is limited. Dotted lines in *Figure 3* indicate this connection. Maybe this phenomenon reflects a feed-back mechanism, caused by ATPases. These membrane-bound ATP-hydrolyzing enzymes are presumably in close association with the ATP cyclase, both acting with ATP as the same substrate, however, with different affinity constants. Numbers 7, 8, 9 and 10 in *Figure 3* show these more indirect points of attack.

Despite the fact that many details are still subject to speculation, the theoretical model explains the molecular mode of action of an ever increasing number of pharmaceutical and physiological agents. It may explain furthermore, why structurally widely different molecules may show the same or a very similar action in the same tissue. For example, lipolysis in fat pads is stimulated by cAMP. This system is blocked by a variety of compounds. The common basis for the antilipolytic effect is the decrease of cAMP level. *Figure 4* (redrawn from Butcher[9]) shows this decrease of epinephrine-stimulated cAMP levels by several antilipolytic compounds. However, the point of attack for these agents is quite different. Insulin and propranolol act on different receptors of the adenyl cyclase system. Prostaglandins influence, very likely, the mechanism in the lipid layer of the membrane. The effects of pyrazole derivatives and of nicotinic acid are not yet completely understood. With insulin, a reaction with the cell surface is sufficient for a cellular response. This has been recently demonstrated by Cuatrecasas *et al.*[10]. Insulin, which had been fixed covalently on a macromolecular carrier (agarose), was fully active towards an adipose tissue culture.

Table 3. Inhibitors of 3′:5′-cyclic nucleotide phosphodiesterases

Methylxanthines[56] (Theophylline, Caffeine etc.)	Phenothiazines[60]
Puromycin[57]	Reserpine[60]
Triiodotyronine[58]	Papaverin[61]
Diazoxide[59]	Triazolo-(4,3-α)-pyrazines[11]
	Sulphonylurea agents[62]

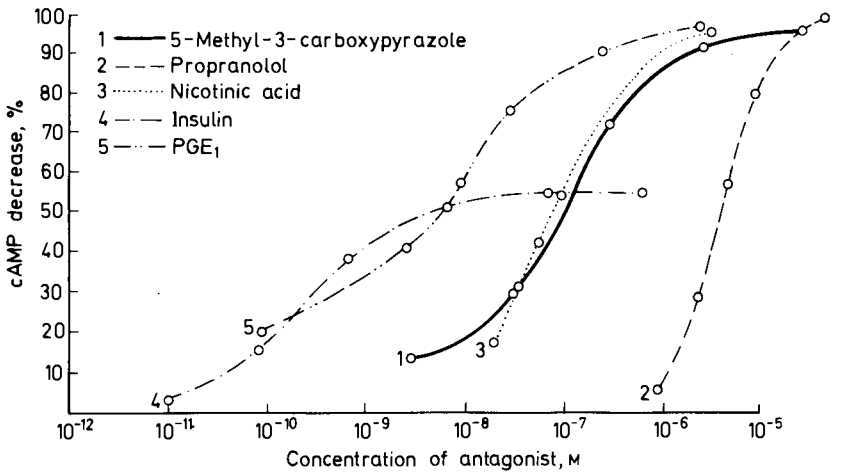


Figure 4. Decrease of cAMP levels under influence of antilipolytic agents in isolated fat cells of the rat (redrawn from Butcher[9]). The term cAMP decrease, % refers to the reduction of the effect of 5.5 μM epinephrine + 1.0 mM caffeine on cAMP levels after 10 minutes incubation with the antagonist.

Inhibition of phosphodiesterases, causing elevation of the cAMP-level is at least partially responsible for the experimentally observed effects of other drugs as shown in *Table 3*. The action of theophylline on the cardiac muscle, of papaverine on the smooth muscle, of phenothiazines on the central nervous system and of sulphonylurea derivatives on the β-cells of the pancreas are some examples of an involvement of PDE in drug action. A good indication is an increase in cAMP following drug administration. Thus it is not surprising that the comparison of the phosphodiesterase inhibition in special tissues with the physiological response *in vivo* plays an increasing role in drug development[11–13].

cAMP-ANALOGUES

Drugs which show effects in the cAMP system should show principally multiple physiological responses. Since, however, those molecules frequently achieve high specificities, a tissue-specific distribution of enzymes of the cAMP-system must exist. These different enzyme patterns, on the other hand, might offer a chance to achieve a selective mode of action by chemical modification of the native second messenger. Experiments along this line are being conducted in several laboratories.

Figure 5 shows the chemical alterations of the cAMP molecule, which have been reported so far. They include the C-2, C-6 and C-8 positions at the heterocyclic moiety, the *N*-glycosidic linkage, the substitution on the 2′-hydroxyl-site of the ribose and the substitution of the oxygen in the P-O-linkages by —S— or —CH_2—. The ability of these derivatives to mimic the various effects of endogenous cAMP was checked in *in vitro* and

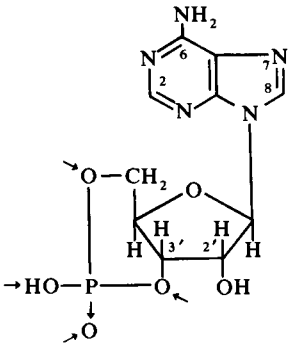


Figure 5. Synthesized derivatives of cyclic AMP. Modifications of the purine base, sugar, phosphate ring and *N*-ribosidic linkage.

in vivo systems. It could be demonstrated that chemical variation of the cAMP molecule produces remarkable changes in the biological activity profile.

This line of investigation originated from a derivative, N^6,2′-O-dibutyryl-cAMP, which was developed by Posternak *et al.*[14]. Several authors have shown that the spectrum of effects of this derivative is similar to cAMP in most, but not in all systems, while the intrinsic activity—when measured in whole tissue preparations or *in vivo*—is much superior. It has been concluded that the increase in potency is due to the lipophilic character and, therefore, improved penetration properties. Moreover, this compound showed a much better resistance to enzymatic attack by phosphodiesterases. Later on it was shown, that increased activity was mainly due to the N^6-monobutyryl residue, since the dibutyryl derivative loses its 2′-O-butyryl-residue under physiological conditions quite rapidly[15].

Measurement of sensitivity to PDE is of major importance in evaluating potential usefulness of cAMP-analogues. *Table 4* shows the rate of hydrolysis

Table 4. Substrate specifity of phosphodiesterase from various tissues. (Collected data from various authors.) The figures represent percentage values of the splitting rate of cAMP

	Brain			Heart		Liver
	Rat	Bovine	Rabbit	Dog	Beef	Rat
Purines						
cyclic AMP	100	100	100	100	100	100
cyclic GMP	70	20	33	33	50–100	49
cyclic IMP	—	30	—	55–65	70–120	86
cyclic XMP	—	—	—	—	15–20	—
cyclic dAMP	—	—	—	—	ca.60	—
Pyrimidines						
cyclic UMP	30	2	11	12–15	5–8	8–9
cyclic CMP	—	0	0	0	0.6	0–5
cyclic dTMP	—	0	—	—	0.4–0.6	—

of some cyclic nucleotides by phosphodiesterases, which were isolated from a variety of species and tissues. With the exception of cUMP, the cyclic pyrimidine nucleotides are not attacked. Recently a cUMP-specific phosphodiesterase was described, but its physiological significance is unknown so far[16]. Based on currently available data, one may conclude that the enzymology of phosphodiesterase is very complex. This enzyme is not only found in the cytoplasm, but also in a particle bound form. Enzymes, derived from different species and from various tissues of the same species, apparently differ in many respects. Even from the same tissue, at least two forms can be identified, which differ in their molecular weight and the K_A-values for cAMP and cGMP. The rate of hydrolysis of these two substrates is influenced by their concentration ratio[17].

In our laboratory, phosphodiesterase from bovine heart was investigated in more detail[18]. In the course of the purification of that enzyme, fractions with discernible activities towards cAMP and cGMP were isolated. In *Table 5*, they have been named PDE-A and PDE-G. We checked the enzymatic activity in these fractions with a series of analogues. The rate of hydrolysis is given in per cent values relative to cAMP hydrolysis. When cAMP derivatives were tested, the relative rates of hydrolysis with both fractions were the same. When, however, the C-6 amino group on the nucleobase was replaced by a C-6 hydroxy group, that is, when derivatives of cIMP and cGMP were subjected to incubation with PDE-A and PDE-G, pronounced differences in the relative rates of hydrolysis were observed. As can be seen from *Table 5*, the decisive factor whether a derivative behaves as an A- or as a G-type is apparently the substituent in the C-6 position of the molecule. Attachment of bulky substituents causes a decrease in the

Table 5. Splitting rates with two fractions of bovine heart PDE relative to the splitting rate of cAMP = 100 per cent[18]. Figures in parentheses represent the splitting rate relative to cGMP = 100 per cent. For more details see the text

Compound	Splitting rate with beef heart PDE, % PDE-A	PDE-G
cAMP	100	100
cIMP	56 (104)	135 (99)
cGMP	54 (100)	136 (100)
cdAMP	59	63
cXMP	20 (37)	44 (33)
2′-*O*-Acetyl-cIMP	62 (114)	120 (88)
2′-*O*-Benzoyl-cIMP	52 (96)	109 (80)
6-Cl-purine riboside cMP	21 (40)	50 (37)
6-(3′,4′-Dimethoxyphenylethyl)-cAMP	0.3 (0.55)	0.7 (0.5)
6-Dimethyl-cAMP	16.7	18.7
N^2-Benzoyl-cGMP	30 (55)	68 (50)

hydrolysis rate. Summarizing our experiments with PDE, the susceptibility to enzymatic attack depends on the chemical structure as follows (*Figure 6*). The highest resistance is observed with 8-substituted analogues, the corresponding C-6 and C-2 derivatives are less resistant[19]. The high biological stability of the 8-substituted cyclophosphate analogues is at least partially

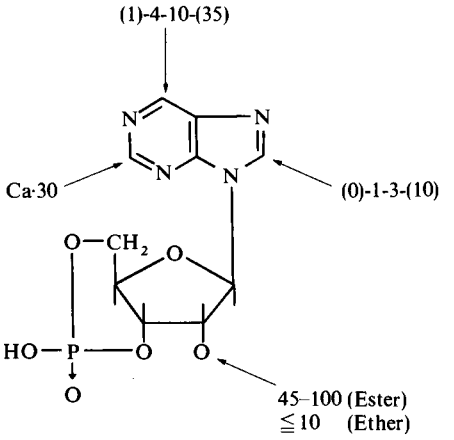


Figure 6. PDE-Splitting rates of derivatives with large substituents[18,19]. The figures indicate percentage of the splitting rate relative to cAMP or cGMP (= 100%), provided there is a bulky substituent at the locations indicated. Figures in parentheses are extreme values, other figures represent frequent values.

caused by the restricted rotation at the *N*-glycosidic linkage (*Figure 7*). The so-called *syn*- and *anti*-conformations differ in the relative position of purine base to the sugar moiety. It appears that the *syn*-form is more resistant to attack by PDE. In addition, the imidazole ring of the purine moiety seems to be a prerequisite for the binding to the active site on the enzyme, in view of the resistance of pyrimidine nucleotides to enzymatic attack. Analogues modified at the C-6 and C-2 position are not restricted in rotation around the *N*-glycosidic linkage and, therefore, are split by phosphodiesterase.

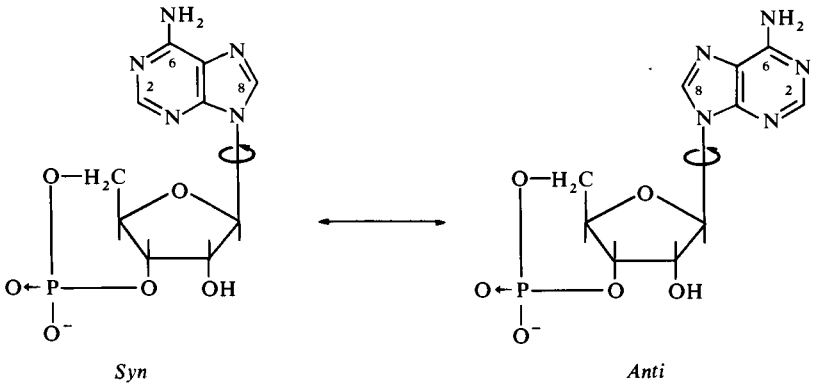
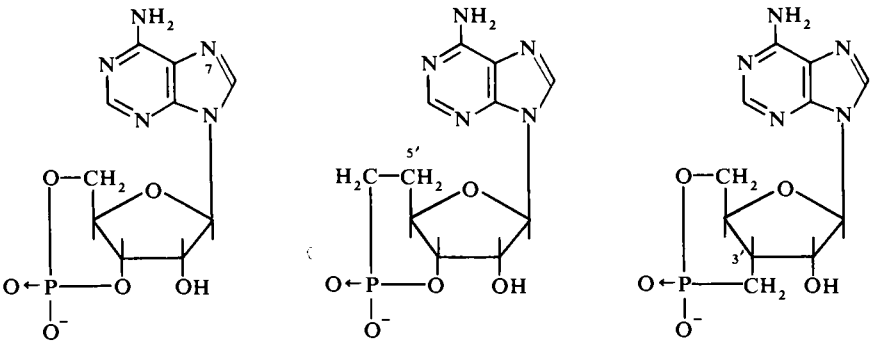


Figure 7. Syn- and *anti*-forms of cAMP. The conformations differ in the relative position of purine base to the sugar moiety. The *syn*-form seems to be less attacked by PDE.

Kuo and Greengard[20] were the first to show that analogues of cAMP may act on protein kinases. Among other compounds, they tested the cyclic phosphate of tubercidine (an antibiotic, developed by the Upjohn Co., in which the N of position 7 is substituted by —CH=), and also the 5′- and the 3′-methylenephosphonate of cAMP, synthesized in the laboratories of the Syntex Co. (*Figure 8*). They used protein kinases from different tissues,



Cyclic TuMP

5′-Methylene phosphonate analogue of cyclic AMP

3′-Methylene phosphonate analogue of cyclic AMP

Figure 8. Structures of analogues of cyclic AMP. The compounds have been developed in the laboratories of the Upjohn Co. (cyclic TuMP) and of the Syntex Co. (methylene cyclic phosphonates).

including a cAMP-specific as well as a cGMP-specific enzyme, both from lobster muscle. The maximum of activity of these derivatives was found to be at about 10^{-6}M. *Table 6* shows selected data from this paper. The tubercidine derivative stimulates all protein kinases in a similar way as cAMP

Table 6. Stimulation of cyclic AMP- and cyclic GMP-dependent protein kinases by cyclic AMP analogues (Selected data from Kuo and Greengard[20]). Protein kinase activity was assayed by measuring the phosphorylation of histone; one unit is defined as that amount of enzyme that transfers 1 pmole of ^{32}P from γ-^{32}P-ATP to recovered protein in 5 min at 30°C

Cyclic nucleotide $5.0 \cdot 10^{-6}$M	Enzyme source and units of activity				
	Bovine		Rat	Lobster	
	Brain	Heart	Adipose cells	Muscle cAMP-depend.	Muscle cGMP-depend.
None	8.6	30.1	9.6	13.4	12.2
Cyclic AMP	135.4	142.2	38.1	45.6	34.1
Cyclic GMP	85.9	124.3	31.8	39.8	43.7
Cyclic TuMP	131.4	141.2	38.5	43.9	42.5
5′-Methylene analogue of cyclic AMP	27.9	142.3	17.1	35.2	13.3
3′-Methylene analogue of cyclic AMP	9.0	35.1	8.7	11.7	11.7

itself. The 5′-methylenephosphonate is active on the kinase from heart muscle, whereas the cGMP-specific enzyme from lobster muscle is not affected. The cyclic 3′-methylenephosphonate is not active in these systems.

The effects of several 8-substituted cAMP derivatives on a protein kinase from bovine brain have been investigated in the Squibb Institute for Medical Research[21]. Very different effects have been found in this system, too. The data of *Table 7* represent the extreme values. Whereas the 8-methylthio-cAMP is even more effective than the second messenger, the 8-ethanol-amino-cAMP is not active.

Table 7. Activation of bovine brain protein kinase by 8-substituted cyclic AMP analogues. (Selected data from Muneyama *et al.*[21])

Analogue	Ratio of activity* in the presence of analogue to activity in the presence of cAMP at the indicated concentration. Concentration M		
	10^{-8}	10^{-7}	10^{-6}
cAMP	1	1	1
8-SCH_3	2.4	1.10	0.94
8-Br	0.73	0.65	0.93
8-$N(CH_3)_2$	0.43	0.56	0.91
8-$NHCH_2C_6H_5$	0	0.091	0.56
8-$NHCH_2CH_2OH$	0	0	0

* Activity measured as pmoles of ^{32}P incorporated into histone.

The different behaviour of cAMP analogues towards protein kinase from different tissues is the molecular basis for the effects in more complicated metabolic processes. In the following chapters, a brief discussion of the current knowledge of cAMP-function in the different systems, the effect of analogues on enzymes in cell-free systems, on isolated tissues and finally *in vivo* is presented. For simplification, statistical data shown in the original papers have been omitted.

GLYCOGENOLYSIS

The molecular mechanisms, by which the cAMP stimulates the transformation of liver and muscle glycogen into glucose are well known due to the work of Sutherland, Krebs and of Greengard (*Figure 9*). A cAMP-dependent protein kinase activates by phosphorylation another kinase (phosphorylase kinase), which, in turn, converts the inactive phosphorylase-b into the active phosphorylase-a by phosphorylation with ATP. Simultaneously, the glycogen synthetase is inhibited by phosphorylation with the same protein kinase which initiates the glycogenolysis. The biological significance of this enzyme cascade lies in the extreme amplification of the hormonal input signal. The concentration of hormones in the blood stream is very small (about 10^{-11} M for peptide hormones, and somewhat higher for catecholamines). This signal of the first messenger is already amplified by

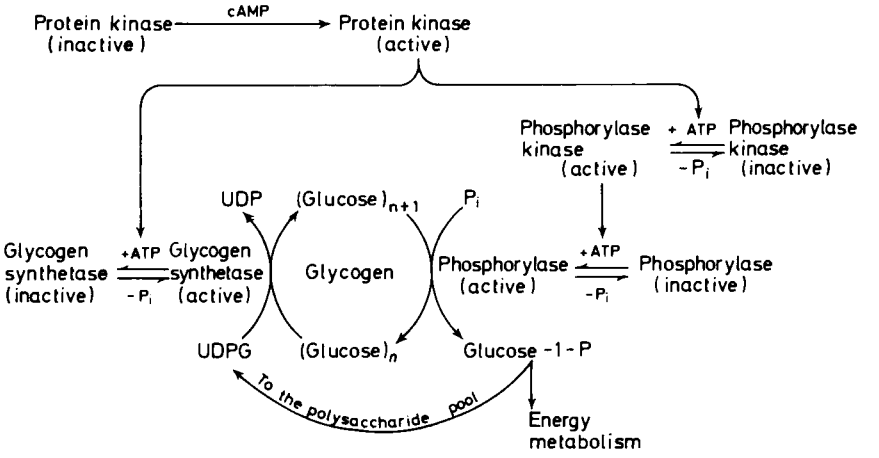


Figure 9. Cyclic AMP and glycogenolysis. The cAMP-dependent protein kinase activates by phosphorylation another kinase (phosphorylase kinase), which, in turn, converts the inactive phosphorylase into the active form. The glycogen synthetase is simultaneously inhibited by phosphorylation with the same protein kinase.

several orders of magnitude at the membrane. One can see therefore, how minimal amounts of a hormone may produce considerable conversion of metabolites.

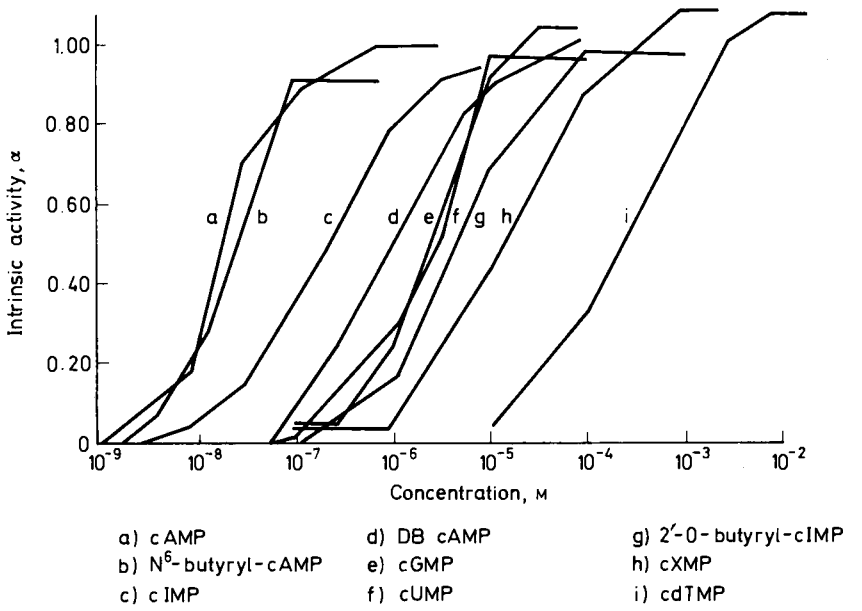


Figure 10. Glycogenolytic effects of cyclic nucleotides[22]. Effects were measured in the 100000 g supernatant of the liver homogenate from the rat.

In our laboratories, a large number of analogues were investigated with respect to glycogenolytic activity in supernatent from muscle or liver homogenate[22,23] (*Figure 10*). Here we found a general stimulation of glycogenolysis, caused by very different concentrations. The dose responses vary by a factor of about 10^4. In spite of this, all compounds display the same maximal activity. This structure–effect relationship is shown in *Figure 11*. The question, whether analogues act via inhibition of phos-

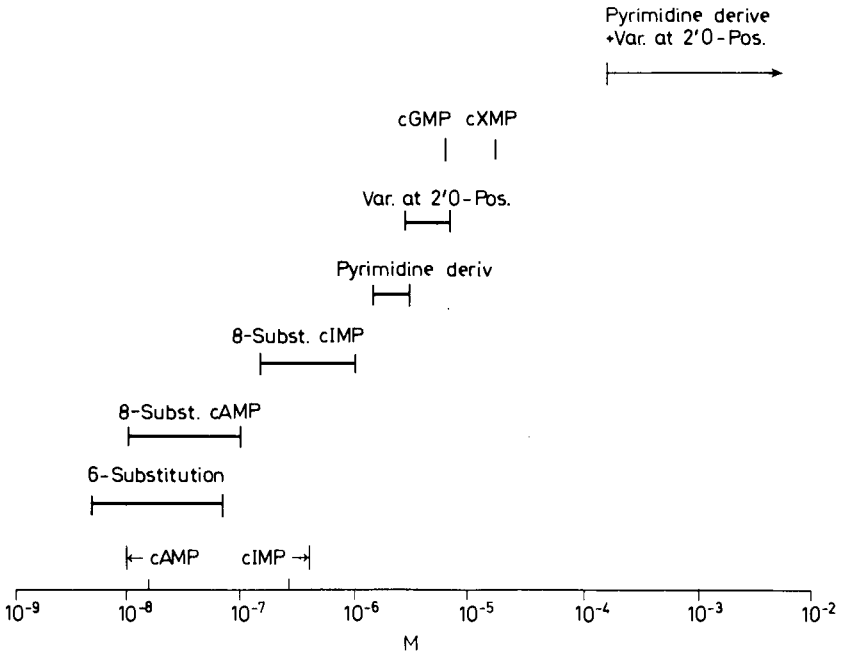


Figure 11. K_A Values of glycogenolytic activity of cyclic nucleotides in a centrifuged liver homogenate[23].

phodiesterase or via activation of protein kinase, may be answered by a comparison of the inhibitor constants of phosphodiesterase and the activation constants of glycogenolysis. As far as has been measured, the K_I-values are two to three orders of magnitude greater than the K_A-values, at least in the case of the 6-substituted analogues. Thus, the activation of glycogenolysis is determined by the protein kinase. These measurements were obtained in the liver system, but the data in skeletal muscle are almost identical. In the measurements made, the protein kinase was the rate limiting step. Contrary to the situation in other tissues, the protein kinases that activate glycogenolysis in liver and muscle appear to be similar.

Different authors investigated the glycogenolytic and gluconeogenetic effects of cAMP and DBcAMP in the perfused liver of the rat and found very strong stimulation, even in intact tissue. This was especially true, when the dibutyryl derivative was used[24–26].

Paoletti *et al.*[27] have checked many analogues, prepared in our laboratories, for glycogenolytic effects *in vivo*. A selection of results is shown in *Table 8*. The figures demonstrate an increase of blood sugar 30 minutes after i.p. administration of 20 and 80 mg kg^{-1} of the cyclic nucleotides. While the cell-free system represents the activity of the cyclic nucleotide *per se*, the *in vivo* system also depends on the penetration ability, the metabolic stability, the excretion pattern and, furthermore, on enzymatic counter-regulation. The activation constants of the *in vivo* system can be roughly estimated or extrapolated and may be correlated to the *in vitro* system. Here it can be shown that even with phosphodiesterase-resistant derivatives, a 1000-fold concentration of the *in vivo* experiments is required to obtain the same effects as those of the *in vitro* experiments. This difference appears to depend mainly on the penetration gradient[27a].

Table 8. Increase of blood glucose (+mg ml^{-1}) 30 minutes after i.p. administration of cyclic nucleotides in the rat. (Selected data from Paoletti *et al.*[27])

Compound	Dose (mg kg^{-1})	
	20	80
DBcAMP	0.79	0.88
DBcGMP	0.73	1.06
2'-O-Butyryl-cIMP	0.00	0.01
N^6-(3',4'-Dimethoxyphenylethyl)-cAMP	1.27	1.28
N^6-(4'-Methylbenzyl-cAMP	1.56	1.92
8-Br-cAMP	0.39	0.58
8-Br-cIMP	0.48	0.91
8-Br-cGMP	0.17	0.31
2-Benzylamino-cIMP	—	0.21
Carboxyethyl-AICAR-cMP	0.08	0.09

The spacing in *Table 8* separate the various groups of analogues. The first group shows the dibutyryl derivatives as reference compounds. In the second group, the strong glycogenolytic activity of the N^6-substituted cAMP-derivatives is striking. Interesting are the only slightly elevated values of the 8-bromo-derivatives and the lack of blood sugar elevation by 2-substituted derivatives, as shown in the last group. Most of the compounds mentioned here are resistant towards hydrolysis with phosphodiesterase.

STEROIDOGENESIS

Trophic hormones of the anterior pituitary gland stimulate steroid synthesis in the adrenal cortex and in the gonads. This procedure is mediated by cAMP. It is likely that the cyclic nucleotide stimulates the oxidation of the cholesterol side chain, thus forming pregenolone as the common precursor of steroid hormones. The effect of the trophic hormones and of cAMP may be inhibited by puromycin, but not by actinomycin. Thus, the secretion of

steroids is preceded by a protein synthesis, which apparently is hormonally controlled at the translation step. In correlation with this, a cAMP-activated protein kinase, which phosphorylates ribosomal proteins selectively, was found in the adrenal cortex[28]. The specificity of the receptor protein of the kinase was investigated. The results are shown in *Figure 12*. One can see the

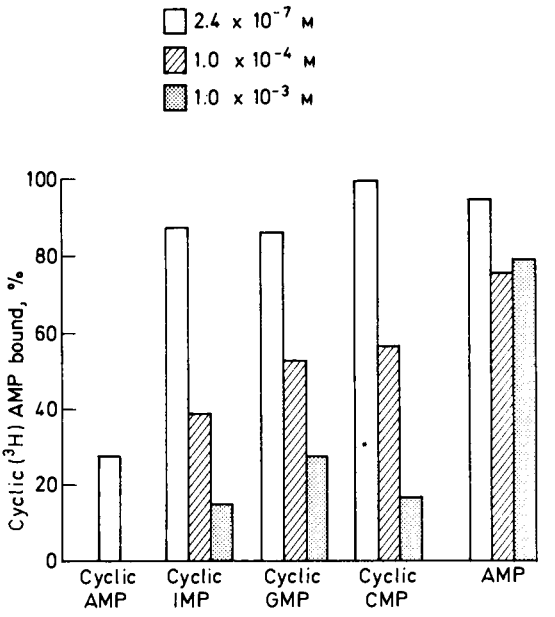


Figure 12. Effects of cyclic nucleotides on the binding of ^{3}H-cAMP to the protein kinase from adrenal cortex[28]. As a 100 % control, binding of the ^{3}H-cAMP without the addition of unlabelled nucleotide is used. For more details, see the text.

effects of various concentrations of cyclic nucleotides on the binding of tritiated cAMP. As a reference, the counts resulting from 100 per cent binding of ^{3}H-cAMP are used. While (non-cyclic) AMP is almost without influence, the cyclic nucleotides of inosine and guanosine compete for the receptor site of the protein kinase, yet only with about 1 per cent of the effectiveness of cAMP.

The steroidogenic effects of these analogues have been compared with the acylated, lipophilic derivatives in slices of adrenal cortex tissue of the rat. DBcAMP was shown to be the most effective one, showing K_A-values two orders of magnitude lower than the other derivatives[29].

cAMP and, even more, DBcAMP have been found to stimulate not only glucocorticoids but also aldosterone synthesis[30]. The K_A-values were compared with ACTH (*Table 9*). cGMP and DBcGMP have been found to stimulate the synthesis of glucocorticoids at 10^{-3}M without any effect on aldosterone secretion. The activation of steroidogenesis in adrenal preparations by 8-substituted derivatives of cAMP was investigated in the Squibb

Table 9. K_A-Values for ACTH or DBcAMP as stimulators of corticosteroidogenesis[30]. About the same concentrations of the effectors stimulate the synthesis of all three corticosteroids

	ACTH	DBcAMP
Aldosterone	$1.3 \cdot 10^{-7}$M	$1.9 \cdot 10^{-5}$M
Corticosterone	$2.4 \cdot 10^{-7}$M	$3.5 \cdot 10^{-5}$M
Cortisol	$3.5 \cdot 10^{-7}$M	$3.0 \cdot 10^{-5}$M

Institute for Medical Research[31]. Some selected data are shown in *Table 10*. The concentrations required for half-maximal secretion were compared. The 8-thiomethyl-cAMP has been shown to be the most active derivative.

Table 10. Steroidogenic response of rat adrenal cell preparations to 8-substituted derivatives of cyclic AMP[31]. The A_{50}-concentration (K_A) is defined as the concentration which stimulates a cell preparation to 50% maximum activity

Compound	Adrenal A_{50} (μM)
cAMP	3300
Dibutyryl-cAMP	95
8-OH-cAMP	90
8-Br-cAMP	85
8-SCH_3-cAMP	65
8-SH-cAMP	380
8-$NHCH_3$-cAMP	460

Paoletti *et al.*[27] measured the effects of a series of derivatives, synthesized in our laboratories, in rats *in vivo*. The increase of corticosterone 30 minutes after i.p. administration of the cyclic nucleotide is given in micrograms per ml (*Table 11*). For comparison, ACTH shows a maximum increase of about 0.50 μg ml^{-1} in this system.

Table 11. Increase of blood steroids (+ μg ml^{-1}) 30 minutes after i.v. administration of cyclic nucleotides in the rat. (Selected data from Paoletti *et al.*[27]). For more details see the text

Compound	Dose (mg kg^{-1}) 20	80
DBcAMP	0.01	0.32
DBcGMP	0.01	0.17
O-2′-Butyryl-cIMP	0.09	0.24
N^6-Benzoyl-cAMP	0.07	0.29
N^6-(3,4-Dimethoxyphenylethyl)-cAMP	0.34	0.39
8-Br-cAMP	0.05	0.09
8-Br-cIMP	0.09	0.44
8-Br-cGMP	0.05	0.17
2-Benzylamino-cIMP	—	0.27
Carboxyethyl-AICAR-cMP	0.13	0.32

As before, the spacings separate the different groups of compounds. The first group shows butyryl derivatives of cAMP, cGMP and cIMP. Only at a high dose of 80 mg kg^{-1} does DBcAMP show a strong effect and butyryl-cIMP a moderate one. Very high activity is shown by the dimethoxy-phenylethylamino-cAMP, which even induces steroidogenesis at 5 mg kg^{-1}. Activity is also exhibited by 8-bromo-cIMP, a compound neither active in cardiovascular systems nor in glycogenolysis.

Also of interest is the imidazol derivative, carboxyethyl-AICAR-cMP (bottom line), which has a pronounced steroidogenic effect. As was shown in our laboratory, these AICAR-derivatives are strongly antilipolytic, too. This will be discussed in the following section.

LIPOLYSIS

The lipolysis of the adipose tissue is assumed to be a key process of metabolism in mammalian system, just as important as glycogenolysis. It is stimulated by several hormones, triggering an enzyme cascade as shown in *Figure 13*[32]. The hydrolysis of the first fatty acid ester in the triglyceride

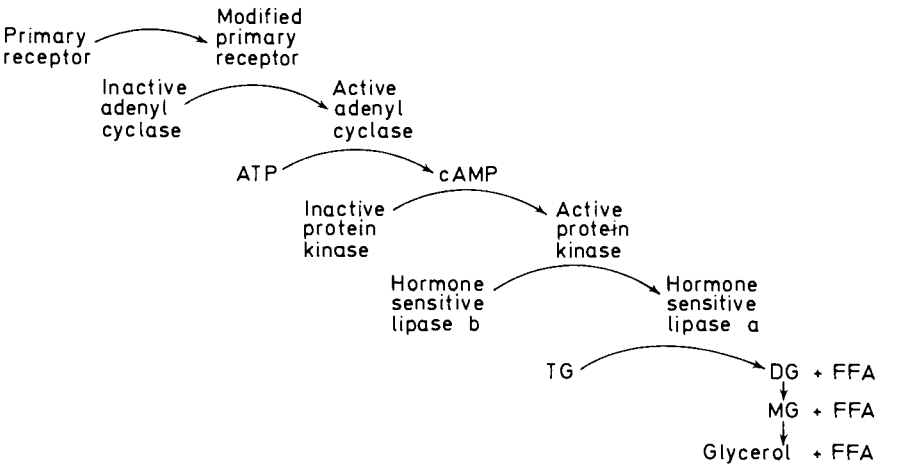


Figure 13. The lipolytic cascade[32]. The rate limiting step is the activation of the hormone sensitive lipase.

has been demonstrated to be the rate limiting step. It is catalyzed by a hormone-sensitive triglyceride lipase. In the laboratories of Krebs and Steinberg, the activation of this lipase by cAMP-dependent protein kinase was found[32,33]. The understanding of the whole system is complicated by a sensitivity to several hormones, by the cooperation of several lipases and probably by a hormone antagonist, arising in the course of lipolysis[34] (for a review see Ref. 9).

Blecher and coworkers[35] compared the lipolytic activity of several cyclic nucleotides in permeable adipocytes of the epididymal fat pad of the rat. This is shown in *Figure 14*. It is somewhat surprising that, in this system, without the barrier of a plasma membrane, the DBcAMP was found to be the most active derivative. The saturation curves prove, nevertheless, the lipolytic activity of most of the other derivatives, with the single exception of cdTMP. Lipolytic activity was also shown by the cTuMP[36].

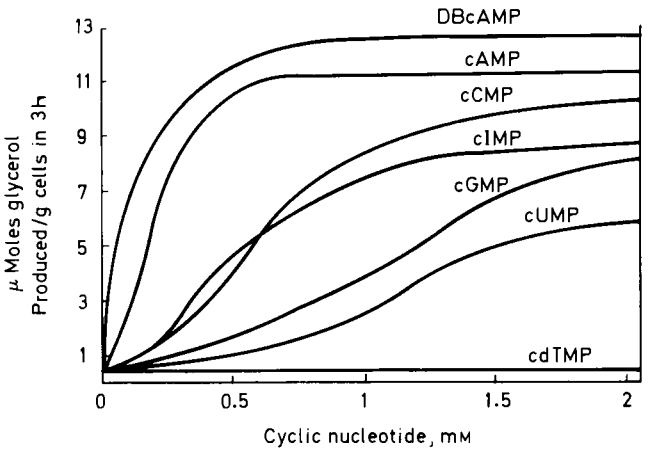
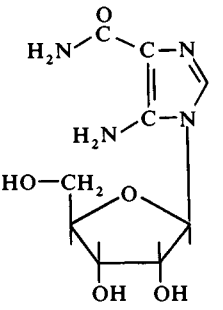


Figure 14. Lipolytic rates in permeable adipocytes as a function of the concentration of analogues of cyclic AMP[35]. Basal lipolysis is shown by the starting point of the curves.

The effects of several 6-, 8- and 2′-substituted derivatives have been investigated both in the Squibb Institute for Medical Research[31] and in our laboratories. The 8-thiomethyl-cAMP, the 8-benzylamino-cAMP and the 8-benzylamino-cIMP have been found to be the most active lipolytic derivatives in the permeable adipocyte system. The effect of the DBcAMP was not surpassed in any case.

An interesting behaviour in the lipolytic system has been found with ribotides of the 5-amino-imidazole-4-carboxamide (AICAR). Its cyclic phosphate and, even more, its 5′-monophosphate have a strong antilipolytic activity, both in the cell-free system and in the isolated adipocyte, as shown in *Figure 15*. This is contrary to the effects of most of the other cyclic nucleotides. AICAR itself is without any effect in this system[37].

There are, nevertheless, derivatives of adenosine, which are strongly antilipolytic, e.g. the N^6-isopropyl-ASN (PIA), studied by Dietmann *et al.*[38]. It acts very likely via an inhibition of the cyclase, as was shown by Westermann *et al.*[39]. In *Figure 16*[9] is shown the dramatic decrease of the intracellular cAMP-level after treatment of rat adipocytes with PIA, the lipolysis being prestimulated by hormones. AICAR-derivatives seem to affect (at least partially) the protein kinase[37].



AICAR

	M	AICAR-3′:5′-MP	AICAR-5′-MP	AICAR
Depression of DBcAMP stimulated lipolysis (cell-free system)	5.10^{-8}	−4%		
	1.10^{-7}		−4%	−4%
	1.10^{-6}		−100%	−2%
	5.10^{-6}	−43%		
	1.10^{-5}		−100%	−3%
	5.10^{-5}	−43%		

Figure 15. Antilipolytic effects of AICAR-deratives in adipocytes and in the cell-free systems from fat-pads of the rat (selected data from Michal *et al.*[37]).

It is noteworthy, that neither cAMP nor most of its derivatives which have been measured so far, showed any lipolytic activity *in vivo*. On the contrary, there was a slight antilipolytic response of DBcAMP[40]. The reason for this may be a counter-regulation, but nevertheless, its mechanism is not yet understood.

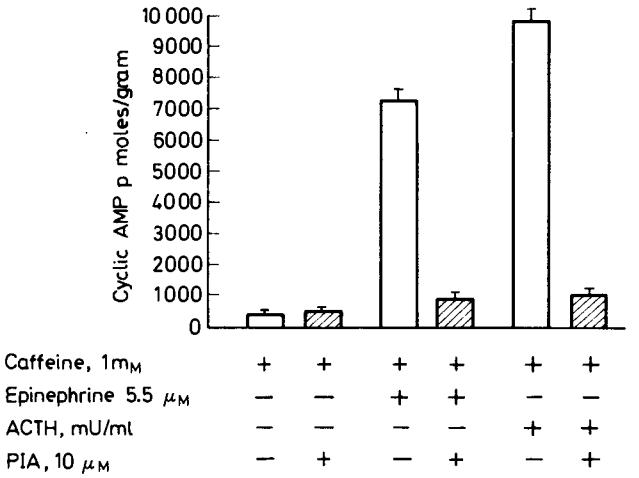


Figure 16. Effects of PIA on cyclic AMP levels in fat cells incubated with epinephrine or ACTH[9]. PIA lowers cAMP levels in fat cells at concentrations as low as 0.1 micromolar. No effects have been observed with broken cell preparations.

SECRETION OF PITUITARY HORMONES

The secretion of the hormones of the pituitary gland is probably regulated by factors released from the hypothalamus. Releasing factors for pituitary hormones have been purified from hypothalamus extracts. They enter the pituitary gland via a portal system and activate cyclases in specific areas of the anterior lobe. As was shown by Labrie *et al.*[41], cAMP stimulates the hormone secretion as well as the protein synthesis in pituitary tissue.

The substrates of a cAMP-dependent protein kinase (purified from pituitary anterior lobe) were found in the rough microsomes, in the secretory granules and in the plasma membrane. These effects are summarized in the scheme proposed by Labrie *et al.*[41] (*Figure 17*).

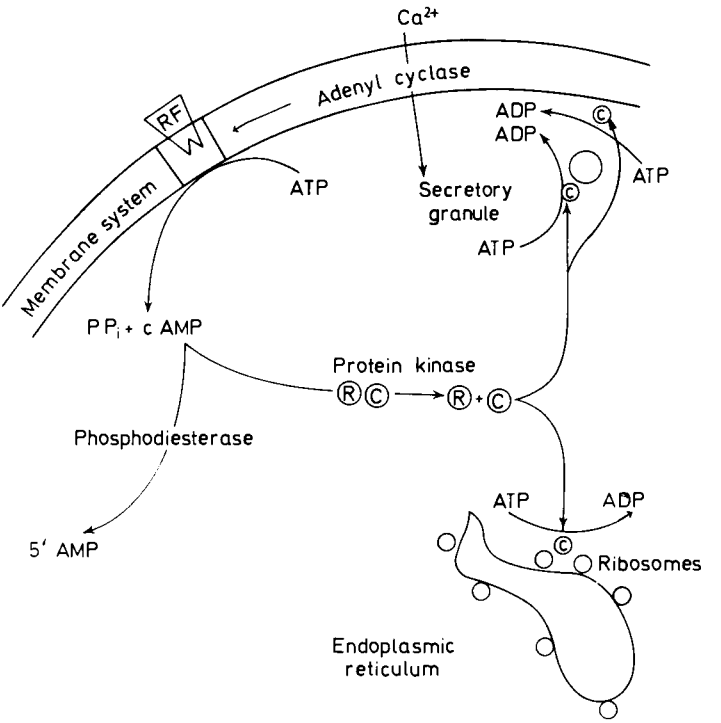


Figure 17. Cyclic AMP response in the anterior lobe of pituitary gland[41].

Very likely, there are similar effects of cAMP in other secretory glands, too. The Michaelis constants of the protein kinases have been found to be about 2.5×10^{-8}M. Concentrations of 10^{-4}M of cAMP or some 8-substituted analogues are inhibitory, very likely due to a competition with ATP at the catalytic subunit of the enzyme[42].

The stimulating effect of some 6-, 8- and 2-substituted derivatives of cAMP on secretion of thyroid stimulating hormone and of growth hormone

has been investigated by Posternak and Cehovic[43]. The authors used slices of anterior pituitary gland of rats. A manifold increase of the effect of the second messenger has been achieved with some of these derivatives, especially with the N^6,2'-O-dibutyryl-8-thio-cAMP. This effect exceeds greatly even the effect of DBcAMP (*Figure 18*).

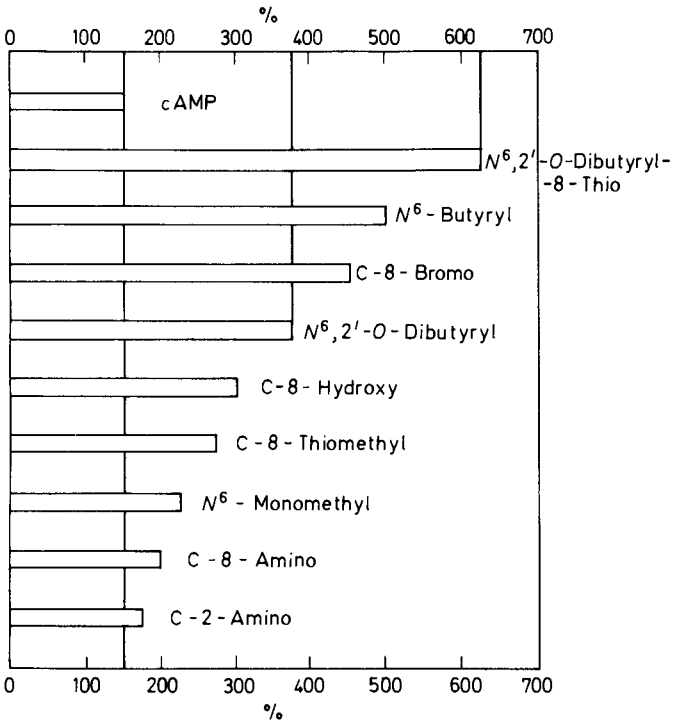


Figure 18. Action of 6-, 8-, and/or C-2-substituted derivatives of cyclic AMP on the release of GH from the anterior lobe of pituitary gland of rats (selected data from Posternak and Cehovic[43]).

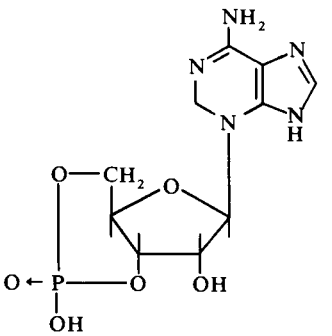


Figure 19. Iso-cyclic AMP[44].

Another interesting derivative which was synthesized and investigated by the same authors was the iso-cAMP, shown in *Figure 19* (Cehovic[44]). Here, the ribotide moiety is shifted from the imidazole to the pyrimidine moiety of the purine molecule. Whereas this compound strongly stimulates the secretion of TSH, it is much less effective in stimulating growth hormone release[43].

EFFECTS OF cAMP-ANALOGUES ON CONTRACTILE TISSUES

The involvement of cAMP in the contraction of vascular smooth muscle of the trachea, ileum, and uterus, has been thoroughly investigated. Although the mechanism is not yet fully understood, the vasoconstrictive response on α-adrenergic stimulation is assumed to be related to a decrease, the vasodilatory response on β-adrenergic stimulation to an increase of the intracellular cAMP level[45]. Because of the large differences of the relative activities of cyclase and phosphodiesterase between the centre and the periphery of the vascular system and because of the different response of the cAMP-system in the various layers of the vascular wall, it is difficult to give a unifying interpretation of drug effects on this system[46].

The perfused, isolated artery of the rat responds to a periarterial electrical stimulation (e.g. catecholamine release) with contractions. The same effect is achieved by drugs, stimulating the vascular tone (ergotamine, imidazole). In this system Berti *et al.*[47] showed a strong relaxation after DBcAMP, whereas cAMP itself has a small, but significant contractive effect. As is shown in *Figure 20*, the 8-bromo-cGMP has a strong relaxing effect on caudal artery which antagonizes both the electrical stimulation and the basal tone[48].

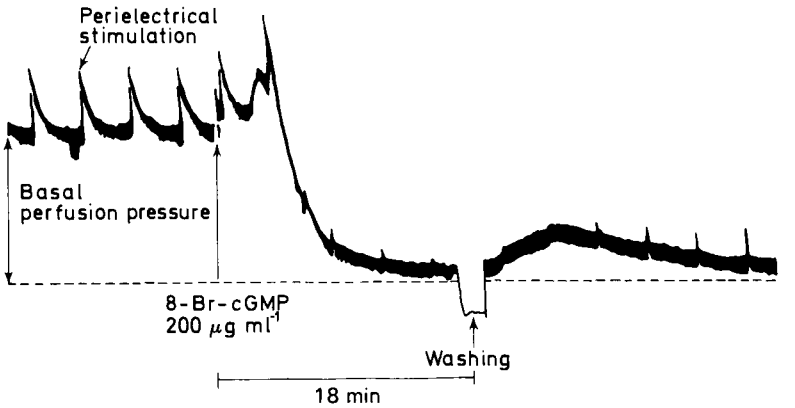


Figure 20. Smooth muscle-relaxing activity of 8-Br-cyclic GMP on the caudal artery of rats[47]. The relaxing activity abolishes both the perielectrical stimulation and the basal perfusion pressure.

The GTP-cyclase is most active in the lung (rats), where the activity is tenfold higher than in other tissues[49, 50]. There may be a special function of cGMP in lung tissue, probably connected to the phosphodiesterase system. This was shown by several authors[51, 52]. Szaduykis-Szadurski *et al.*[53] investigated the effect of several analogues of cAMP and cGMP on the smooth muscle of the isolated trachea of guinea pig. In this system too, 8-bromo-cGMP was the most active derivative. A comparison of the effects of 8-bromo-cGMP, DBcAMP and DBcGMP is shown in *Figure 21* (redrawn

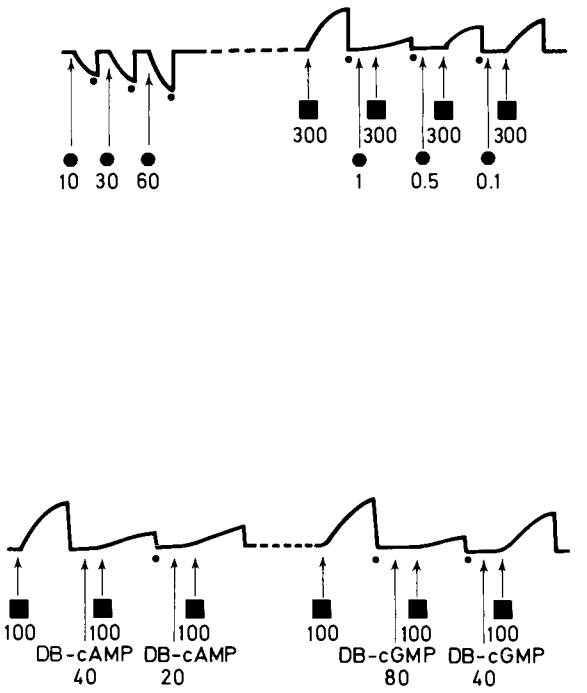


Figure 21. Effect of 8-Br-cGMP (●) on basal tone (left) and on the contraction effect of imidazole ■ (right) of tracheal smooth muscle from guinea pig (upper graph) compared with the effect of DBcAMP and DBcGMP (lower graph)[48]. Numbers represent the final concentration of the drugs in $\mu g\ ml^{-1}$.

from Szaduykis-Szadurski and Berti[48]). The upper left graph shows the dose-dependent smooth muscle relaxing effect of 8-bromo-cGMP. The upper right graph demonstrates the effect on imidazole-contracted muscle, which is antagonized by the cGMP derivative. The lower graph shows the corresponding effects of the dibutyryl derivatives, which antagonize imidazole only at 20-fold to 80-fold higher concentrations than 8-bromo-cGMP. Berti[54] demonstrated in *in vivo*-experiments that 8-bromo-cGMP relaxed histamine-induced bronchospasms in the guinea pig twice as effectively as theophylline. It should be emphasized that neither 8-bromo-GMP nor 8-bromo-guanosine showed any effect in either of these systems.

At the Squibb Institute for Medical Research, several 8-substituted cAMP-derivatives were compared with theophylline in a similar tracheal system[55]. Selected data are shown in *Table 12*. The most effective compound was 8-benzylthio-cAMP, being almost 10 times more active than DBcAMP. The 8-bromo-cAMP was almost as active as DBcAMP. Similar data were obtained with the portal vein of the rat. None of these derivatives, however, reached the response of theophylline (which was surpassed, as shown above, by 8-bromo-cGMP). This compound, therefore, reveals a pronounced relaxing activity on the vascular smooth muscle.

Table 12. Relaxant concentrations *in vitro* of theophylline and analogues of cyclic AMP on guinea pig trachea. (Selected data from Rubin *et al.*[55]). IC_{50} = average concentration ($\mu g\ ml^{-1}$) producing 50% relaxation of control response

Compound	IC_{50} ($\mu g\ ml^{-1}$) 10 min
Theophylline	4.4 ± 0.4
DBcAMP	247 ± 13
8-SCH_2-C_6H_5-cAMP	23 ± 2
8-SCH_3-cAMP	93 ± 7
8-$N(CH_3)_2$-cAMP	224 ± 33
8-Br-cAMP	236 ± 67

Finally, let us consider the cardiovascular response. The effects of epinephrine and glucagon (the positive inotropic effect on heart muscle and coronary vasodilatation) are accompanied by an elevation of the cAMP level, although the exact mechanism is still under discussion. The response in muscle seems to be closely connected with the mobilization of intracellular pools of calcium. A further system of cyclases, bound to subcellular particles,

Table 13. Effect of analogues of cAMP on blood pressure and heart rate (anaesthetised rats, 20 mg kg^{-1}, i.v.). (Selected data from Paoletti *et al.*[27])

Compound	Blood pressure (mmHg)			Heart rate (min^{-1})		
	0′	3′	36′	0′	3′	36′
DBcAMP	106	80	95	394	330	300
DBcGMP	110	110	115	420	420	420
N^6-(3,4-Dimethoxyphenylethyl)-cAMP	115	38	38	405	120	90
N^6-(4-Methylbenzyl)-cAMP	102	68	75	420	428	322
8-Benzylamino-cAMP	88	83	80	330	320	310
8-Methylmercapto-cAMP	108	97	96	366	402	354
8-(4-Methylbenzylamino)-cIMP	115	115	103	440	430	380
8-Br-cGMP	95	70	80	360	360	360
2-Benzylamino-cIMP	93	91	86	335	357	323
Carboxyethyl-AICAR-cMP	90	87	88	340	330	310

is included in the discussion of the mechanism as well as the effect of another second messenger.

In view of this not yet well understood mechanism of contractile response to cAMP, the measurements of blood pressure and heart rate should be understood phenomenologically only. *Table 13* shows the results obtained with anaesthetised rats[27].

One may correlate the data with those of DBcAMP and DBcGMP, shown in the first group. There is a strong response to compounds with an aralkylgroup in the N^6-position, such as N^6-dimethoxyphenylethyl-cAMP. Similar substituents in the 8-position of cAMP (third group) have no effect. As mentioned above, some of these 8-substituted derivatives (e.g. 8-methylmercapto- and 8-benzylamino-cAMP) have strong metabolic effects in other tissues. Substituents attached to the cIMP or cGMP moieties at the 8- and 2-positions do not cause effects on blood pressure and heart rate, although some of these compounds have steroidogenic effects, while the 8-bromo-cGMP relaxes the vascular smooth muscle, as was shown before.

PHARMACOLOGICAL ASPECTS

Do those findings have any significance for the development of new drugs? Considering the different patterns of the physiological response and the rather high specificity of the effects seen with some of these cAMP-derivatives, one may be optimistic. As we have seen, this specificity distinguishes steroidogenesis, antilipolysis, hormone secretion, the relaxation of vascular smooth muscle, etc. It seems possible to obtain compounds exerting metabolic effects quite free from vasodilatory action. Also, compounds showing effects on the tracheal smooth muscle without affecting the cardiovascular system can be selected. Finally, we have seen that the multivalent response of the second messenger—without higher specificity—is much enhanced in some analogues, e.g. DBcAMP.

As was found by Levine *et al.*, some of the effects of DBcAMP can also be reproduced in humans. The chance of such a multivalent derivative being used as a therapeutic agent depends on whether one can demonstrate that a pathological deficiency is due to a disturbance of the first messenger system. Since our knowledge of pathogenesis is not developed so far, it is too early to prove this point. Compounds with more specific effects appear easier to put to work. Moreover, for the time being, second messenger analogues seem to be a useful tool in the elucidation of basic biochemical mechanisms. This may lead to a better understanding of molecular events in drug effects. In the future, we hope that medicine, as well as biochemistry, will be able to describe normal and pathological behaviour of cellular systems in the common language of molecular biology.

REFERENCES

[1] E. W. Sutherland and T. W. Rall, *Pharmacol. Rev.* **12**, 265 (1960).
[2] G. A. Robison, R. W. Butcher and E. W. Sutherland, *Cyclic AMP*, Acad. Press, New York and London (1971).
[3] J. F. Kuo and P. Greengard, *Proc. Nat. Acad. Sci. U.S.* **64**, 1349 (1969).

[4] P. Greengard, *Annal. New York Acad. Sci.* **185**, 18 (1971).
[5] J. G. Hardman, J. A. Beavo, J. P. Gray, T. D. Carisman, W. D. Patterson and E. W. Sutherland, *Annal. New York Acad. Sci.* **185**, 27 (1971).
[6] W. Y. Cheung, *Biochim. Biophys. Res. Commun.* **38**, 533 (1970).
[7] C. D. Asby and D. A. Walsh, *Feder. Proc.* **31**, 439, Abstr. nr. 1250 (1972).
[8] T. E. Donnelly Jr., J. F. Kuo, E. Miyamoto and P. Greengard, *Feder. Proc.* **31**, 439, Abstr. nr. 1254 (1972).
[9] R. W. Butcher, *Arch. Pharmakol.* **269**, 358 (1971).
[10] P. Cuatrecasas, *Proc. Nat. Acad. Sci. U.S.* **63**, 450 (1969).
[11] A. R. Summerville, M. L. Rabouhans and A. A. Smith, *Biochem. J.* **120**, 11 P (1970).
[12] H. Sheppard and G. Wiggan, *Molec. Pharmacology* **7**, 111 (1971).
[13] B. Beer, M. Chasin, D. E. Clody, J. R. Vogel and Z. P. Horovitz, *Adv. Cycl. Nucl. Res.* **1**, 566. Raven Press, New York (1972).
[14] T. Posternak, E. W. Sutherland and W. F. Henion, *Biochim. Biophys. Acta* **65**, 558 (1962).
[15] N. I. Swislocki, *Analyt. Biochem.* **38**, 260 (1970).
[16] U. Klotz and K. Stock, *Arch. Pharmakol. Exp. Pathol.* **269**, 117 (1971).
[17] G. I. Drummond and M. Yamamoto in P. Boyer, *The Enzymes*, Vol. IV, 3rd ed., p. 596. Academic Press, New York and London (1971).
[18] G. Michal, G. Weimann, M. Nelboeck and C. Thiessen (1973). In preparation.
[19] G. Michal, M. Nelboeck and G. Weimann, *Z. Anal. Chem.* **252**, 189 (1970).
[20] J. F. Kuo and P. Greengard, *Biochem. Biophys. Res. Commun.* **40**, 1032 (1970).
[21] K. Muneyama, R. J. Bauer, D. A. Shuman, R. K. Robins and L. N. Simon, *Biochemistry* **10** 2390 (1971).
[22] M. DuPlooy, G. Michal, G. Weimann, M. Nelboeck and R. Paoletti, *Biochim. Biophys. Acta* **230**, 30 (1971).
[23] G. Michal, M. DuPlooy, M. Woschee, M. Nelboeck and G. Weimann, *Z. Anal. Chem.* **252**, 183 (1970).
[24] L. Menahan and O. Wieland, *Biochem. Biophys. Res. Commun.* **29**, 880 (1967).
[25] J. H. Exton and C. R. Park, *J. Biol. Chem.* **243**, 4189 (1968).
[26] R. A. Levine and S. E. Lewis, *Biochem. Pharmacol.* **18**, 15 (1969).
[27] R. Paoletti, F. Berti, P. F. Spano, G. Michal, G. Weimann and M. Nelboeck, *Pharm. Res. Commun.* (1972). In press.
[27a] G. Michal, *Pharm. Res. Commun.* (1972). In press.
[28] L. D. Garren, G. N. Gill and G. M. Walton, *Ann. New York Acad. Sci.* **185**, 210 (1971).
[29] P. Bieck, H. Vapaatalo and E. Westermann, *Acta Physiol. Scand.* **80**, nr. 4, 28 A (1970).
[30] H. Erbler, *Arch. Pharmakol. Exp. Pathol.* **270**, Suppl. 1, R 26 (1971).
[31] A. C. Free, M. Chasin, V. S. Paik and S. M. Hess, *Biochemistry* **10**, 3785 (1971).
[32] D. Steinberg and I. K. Huttunen, *Adv. Cycl. Nucl. Res.* **1**, 47. Raven Press: New York (1972).
[33] I. D. Corbin and E. G. Krebs, *Biochem. Biophys. Res. Commun.* **36**, 328 (1969).
[34] R. J. Ho and E. W. Sutherland, *J. Biol. Chem.* **246**, 6822 (1971).
[35] M. Blecher, J. T. Ro'ane and P. D. Flynn, *Arch. Biochem. Biophys.* **142**, 351 (1971).
[36] M. Blecher, J. T. Ro'ane and P. D. Flynn, *Biochem. Pharmacol.* **20**, 249 (1971).
[37] G. Michal and G. Jurz, *Pharm. Res. Commun.* (1973). In press.
[38] K. Dietmann, W. Schaumann, F. H. Schmidt and H. Stork, Lecture at the 3rd Intern. Symposium on Drugs Affecting Lipid Metabolism, Milan (1968).
[39] E. Westermann, K. Stock and P. Bieck, *Fettstoffwechsel* **5**, 68 (1969).
[40] P. Bieck, K. Stock and E. Westermann, *Life Sci.* **7**, 1125 (1968).
[41] F. Labrie, G. Pelletier, A. Lemay, S. Lemaire, G. Poirier, N. Barden, G. Beraud, R. Boucher, M. Gauthier and A. DeLean, *Acta Physiol. Hungar.* In press.
[42] S. Lemaire, F. Labrie and M. Gauthier, *J. Biol. Chem.* In press.
[43] T. Posternak and G. Cehovic, *Annal. New York Acad. Sci.* **185**, 42 (1971).
[44] G. Cehovic, *Compt. Rend. Acad. Sci. Paris*, Série D, 2929 (1969).
[45] E. W. Sutherland, G. A. Robison and R. W. Butcher, *Circulation* **37**, 279 (1968).
[46] L. Triner, Y. Vulliemoz, M. Verosky and G. G. Nahos, *Cardiovasc. Res.* **310**, VI. World Congr. Cardiol., Abstr. T (1970).
[47] F. Berti, V. Bermareggi and V. Mandelli, *Arch. Intern. Pharmacodyn.* **192**, 247 (1971).
[48] L. Szaduikis-Szadurski and F. Berti, *Pharmacol. Res. Commun.* **4**, 53 (1972).
[49] J. G. Hardman and E. W. Sutherland, *J. Biol. Chem.* **244**, 6363 (1969).
[50] A. A. White and G. D. Aurbach, *Biochim. Biophys. Acta* **191**, 686 (1969).

[51] F. Murad, V. Manganiello and M. Vaughan, *J. Biol. Chem.* **245**, 3352 (1970).
[52] J. A. Beavo, J. G. Hardman and E. W. Sutherland. *J. Biol. Chem.* **245**, 5649 (1970).
[53] L. Szaduikis-Szadurski, G. Weimann and F. Berti, *Pharmacol. Res. Commun.* **4**, 63 (1972).
[54] F. Berti (1972).(Unpublished).
[55] B. Rubin, E. H. O'Keefe, M. H. Waugh, D. G. Kotler, D. A. DeMaio and Z. P. Horovitz, *Proc. Soc. Exper. Biol. Med.* **137**, 1244 (1971).
[56] R. W. Butcher and E. W. Sutherland, *J. Biol. Chem.* **237**, 1244 (1962).
[57] M. M. Appleman and R. G. Kemp, *Biochem. Biophys. Res. Commun.* **28**, 564 (1966).
[58] L. R. Mandel and F. A. Kuehl, *Biochem. Biophys. Res. Commun.* **28**, 13 (1967).
[59] G. Senft, K. Munske, G. Schultz and M. Hoffmann, *Arch. Pharmakol. Exp. Pathol.* **259**, 344 (1968).
[60] F. Honda and H. Imamura, *Biochim. Biophys. Acta* **161**, 267 (1968).
[61] W. R. Kukovetz and G. Poech, *Arch. Pharmakol. Exp. Pathol.* **267**, 189 (1970).
[62] G. Brooker and M. Fichman, *Biochem. Biophys. Res. Commun.* **42**, 824 (1971).

PRINCIPLES OF NEUROCHEMISTRY AND DRUG SCREENING PROCEDURES IN NEUROPSYCHOPHARMACOLOGY

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ABSTRACT

This presentation surveys modern concepts for drug screening as they have emerged from current neurochemical research. Among others, the concept of multiple forms of MAO and phosphodiesterase has revolutionized the approach to drug screening of compounds that change neuronal function through inhibition of these enzymes. Neurochemical progress has opened new vistas and has given indications on how to screen MAO inhibitors for maximum efficacy while avoiding potential side effects. The finding that drugs acting on postsynaptic receptors can now be screened in preparations with receptors made supersensitive by denervation provides another new model, made possible by the discovery that 6-OH-dopamine and 6-hydroxyserotonin can lesion neurons selectively. The use of turnover rate measurements in association with other tests can contribute valuable information on the anatomical site of action of drugs. These tests have a great predictive value if conducted with quantitative criticism of dose–response relations.

INTRODUCTION

Numerous examples can be marshalled to provide evidence that serendipity has played a major role in the discovery of the most important drugs introduced in neuropsychiatry during the last 25 years. However, sporadic exceptions exist. One of them is the successful use of L-dopa as a symptomatic remedy for Parkinson's disease. Undoubtedly, L-dopa would not have been tested without the important discovery made by Ehringer and Hornykiewicz[1] that a decrease of dopamine concentration in striatum is a symptom of Parkinson's disease. This finding might not have promoted the use of L-dopa as a potential remedy in Parkinsonism[2] had Blashko[3] not shown that this amino acid is involved in the biosynthesis of endogenous catecholamines. When L-dopa was first tested in Parkinsonism it was also known that parenteral administration of L-dopa increases the brain concentrations of dopamine in normal animals and in animals treated with reserpine[4].

A number of drugs presently used in neuropsychiatry were developed

as a result of elaborate programmes of chemical synthesis. Many such programmes evolved from evidence indicating that a given molecular group was responsible for the therapeutic effect of existing remedies. In these attempts such molecular groups were introduced into appropriate chemical structures in order to produce new compounds with a better therapeutic index. Perhaps in these chemical manipulations compounds with unexpected therapeutic properties were obtained, but these properties were not detected because the pharmacological screening of the new molecules was designed to reveal and evaluate the properties of the drug used as a prototype. Thus, our lack of ingenuity in formulating screening procedures has confined many potentially useful drugs to the shelves of drug firms. I am certain that each one of us could mention an appropriate example to support this statement. In fact, a number of drugs have acquired a proper therapeutic indication many years after their synthesis. Often this therapeutic indication is different from that originally intended when these drugs were synthesized. A case in point is α-methyl-dopa which was originally synthesized as a potential hypertensive drug, but when tested failed to cause hypertension. Several years later, α-methyl-dopa was tested clinically as a hypotensive agent because appropriate screening tests[5] had shown that this drug could lower the concentrations of norepinephrine in central and peripheral noradrenergic neurons[6]. Apparently, this was a lucky rationalization for α-methyl-dopa is still used as a hypotensive agent.

Table 1. Possible sites of interactions between drugs and mechanisms regulating synaptic function

Sites of interaction	Drug effects	
	Short term	Long term
Synaptic receptor	Stimulation: Direct / Indirect (inhibition of transmitter metabolism)	Transynaptic regulation of protein synthesis
	Inhibition	
	Interaction with cyclic nucleotides (second messengers)	
Nerve terminal membrane	Interaction with transmitter retrieval	False transmitter storage
	Interaction with transmitter release: Dependent on neuronal activity / Independent from neuronal activity	
Intraneuronal	Interaction with transmitter storage	Axonal transport
	Interaction with transmitter synthesis	
	Interaction with transmitter catabolism	

With these considerations in mind, I have decided to talk to you about certain fundamental principles that are emerging from current neurochemical research. With the progress of our understanding of brain biochemistry, these concepts are acquiring increasing value in devising drug screening

procedures which help us to detect the potential usefulness of new neuropsychiatric drugs.

Table 1 summarizes some regulatory mechanisms of neuronal function which are possible sites for drug action: the receptor, the nerve terminal membrane, and various intraneuronal sites. Each of these sites can participate in short- and long-term regulation of neuronal function: the present report will be mainly concerned with drug action on sites involved in short term regulation of neuronal function. This decision does not imply that long term regulation should not concern pharmacologists: rather it indicates that our knowledge is still too fragmentary to be expressed in suitable models for drug screening.

THE CONCEPT OF POSTSYNAPTIC RECEPTOR IN DRUG DEVELOPMENT

The discovery that 3′,5′-cyclic AMP catalyzes the enzymatic phosphorylation of proteins by kinases[7] and the realization that the function of membrane-bound adenylate cyclase can be regulated by catecholamines[8], including norepinephrine (NE)[9] and dopamine (DM)[10], have prompted a drastic revision in the conceptualization of the biochemistry of postsynaptic receptors. Catecholamines and, perhaps, other putative transmitters, activate the membrane-bound adenylate (or guanylate) cyclase increasing the formation of cyclic nucleotides from ATP and GTP. We do not understand how these nucleotides can elude inactivation by phosphodiesterase, a soluble enzyme present in cytoplasm, to convey intracellularly the message brought to the cell surface by the transmitter. However, the 3′,5′-cyclic AMP (termed second messenger) and the intracellular mechanisms that make its action possible, should now be considered as an important site for the action of drugs that mimic the effects of certain transmitters. Since there are different molecular forms of phosphodiesterases[11], which may be cell specific[12] and possess different sensitivity toward drug inhibition, the ubiquitous adenylate cyclase system may be manipulated pharmacologically with some degree of selectivity. Probably the postsynaptic receptor uses the adenylate cyclase as a transducer to transform the synaptic event into a message for the mechanisms that control protein synthesis in the postsynaptic cell.

1. Receptor stimulants

(*i*) *Direct stimulants*

The studies of cholinergic and noradrenergic peripheral neurons have pioneered the present understanding of synaptology by proving that neurons control the cells they innervate by secreting appropriate chemical mediators at synapses. The secretion of these transmitters enables neurons either to initiate a variety of responses in the postsynaptic cell (contraction of striated muscles, secretion of glands, etc.) or to modulate the frequency of spontaneous rhythmical activities in cells endowed with such a property.

More than thirty years ago, as a corollary to these studies, Dale formulated a basic axiom concerning the chemical nature of synaptic transmitters. This axiom, which is still widely accepted on its intrinsic merit, postulates that

each neuron synthesizes, stores, and secretes one and only one type of transmitter. This concept coupled with the uniformity of responses elicited by a given transmitter in a given peripheral tissue (contraction of striate muscles, secretion of salivary glands, etc.) has contributed to the expectation that, in the brain, drugs which mimic a given transmitter elicit a defined functional and behavioural response. Of course, when such drugs are administered systemically, they elicit complex responses due to stimulation of the numerous specific receptors located in neuronal systems involved in the control of diversified neuronal mechanisms. Such unexpected complexities of drug effects are usually referred to as side effects of the drug. This is a misnomer. These side effects are not collateral and unexpected effects of the drug, but they reflect the stimulation of identical receptors involved in other brain functions. As an example, we may take chlorpromazine and other neuroleptics chemically related to chlorpromazine. It is presently believed that these drugs elicit their action because they occupy dopaminergic receptors where they prevent the action of dopamine[13]. It is, therefore, not surprising that after prolonged administration, the neuroleptic action is contaminated by extrapyramidal disturbances because dopaminergic receptors identical to those necessary for the neuroleptic action are involved in the regulation of extrapyramidal function. Similar considerations apply to the emesis occurring during the administration of L-dopa to Parkinsonian patients: this reflects the involvement of dopamine receptors in the function of the myelencephalic trigger zone, a site of convergence for neuronal activity involved in the control of vomiting.

Table 2. Structures innervated by dopaminergic, serotonergic, and noradrenergic neurons in rat brain

Cell bodies localization	Transmitter stored	Brain structure innervated
Substantia nigra	Dopamine	Limbic forebrain Neo striatum
Locus coeruleus	Norepinephrine	Cerebellum Limbic forebrain Thalamus Hypothalamus
Raphe medianus	Serotonin	Paleostriatum Neostriatum Limbic forebrain Cerebellum Thalamus Hypothalamus Spinal cord

To recapitulate this point, let us consider the data reported in *Table 2* which illustrates the projection of noradrenergic, dopaminergic, and serotonergic neurons to various brain structures. This *Table* includes only three putative transmitters but there is reason to believe that other neuronal systems may display an equally diffuse innervation. This suggests that many

different brain structures are regulated by the same transmitter. Electrophysiological studies on neuronal responses to the application of various transmitters in the vicinity of neuronal cell bodies show that the application of a given putative transmitter elicits very similar responses in various neuronal populations[14] of brain. Therefore, one can summarize, that the effects on the excitability of the neuronal membrane are not different.

In conclusion, a selectivity of neurological and behavioural responses is at best the exception in the pharmacological profile of drugs which directly activate the postsynaptic receptors of a given neurotransmitter. Perhaps when pathological conditions resembling surgical denervation change receptor function, its susceptibility to the action of drugs that mimic the transmitter can be enhanced (denervation supersensitivity). This enhancement results in a certain degree of selectivity of the drug response in the

Table 3. Selective depletion of spinal cord 5-HT by intracisternal injection of 6-hydroxyserotonin (6-HS)*

Brain structure	Dopamine nmole $g^{-1}/\pm SE$		Serotonin nmole $g^{-1}/\pm SE$		Norepinephrine nmole $g^{-1}/\pm SE$	
	Saline	6-HS	Saline	6-HS	Saline	6-HS
Spinal cord	—	—	4.3 ± 0.04	1.8 ± 0.12*	1.9 ± 0.12	2.2 ± 0.12
Medulla pons	—	—	4.9 ± 0.57	5.7 ± 0.21	3.5 ± 0.23	3.5 ± 0.14
Hypothalamus	—	—	9.5 ± 1.5	7.7 ± 0.59	11 ± 0.76	10 ± 1.5
Cerebellum	—	—	1.8 ± 0.14	1.6 ± 0.15	1.6 ± 0.19	1.5 ± 0.08
Telencephalon	3.8 ± 2.6	4.2 ± 2.8	1.9 ± 0.12	1.6 ± 0.22	1.5 ± 0.1	1.6 ± 0.11

* 6HS (0.39 μmole/rat) was injected 14 days before the assay $P < 0.01$

denervated receptors. When the location of the pathological condition affecting receptor function is known and if the nerves afferent to this brain area are accessible, a denervated preparation may be an appropriate model system to screen the efficacy of receptor stimulants. An index of the selectivity of this drug response can be obtained by comparing responses elicited in intact and in denervated preparation. An example of this type of screening is the one currently employed for drugs to be used in Parkinson's disease. In rats the monolateral injection of 6-hydroxydopamine[15] in the *substantia nigra* selectively destroys the cell body of the dopaminergic neurons of the nigro-striatal pathway (*Table 2*). These rats either exhibit circling toward the lesioned side or remain immobile. The injection of drugs that mimic the effects of dopamine on striatal receptors causes intense circling away from the lesioned side. With this preparation, one can have a realistic appreciation of the therapeutic index of the drug in conditions of receptor supersensitivity. Similar screening models can be devised for noradrenergic receptors which can be denervated by injections of 6-hydroxydopamine in appropriate brain regions and for serotonergic receptors which can be denervated by injections of 6-hydroxyserotonin (6-HS)[16]. This drug injected intracisternally destroys serotonergic nerve terminals of spinal cord selectively (*Table 3*).

Since serotonergic function is involved in spinal reflexes, it may turn out that pretreatment with 6-HS may be a suitable model to test drugs that directly stimulate serotonergic receptors.

(*ii*) *Indirect stimulants.*

The term 'indirect stimulation of postsynaptic receptors' describes the effect of drugs which increase the amount of transmitter reaching postsynaptic receptors. These drugs may interfere with various mechanisms regulating the release from nerve endings of the transmitter. These mechanisms are located either in the membrane of the nerve terminal or extraneuronally in the vicinity of the postsynaptic receptors. Here we shall consider only those mechanisms which are extraneuronally located and are enzymatic in nature. These mechanisms have a particular importance for the cholinergic mechanism and influence the noradrenergic function to a much smaller extent. The two enzymes involved are termed acetylcholinesterase and catecholamine-*O*-methyltransferase. Several inhibitors of cholinesterases have been developed. Due to the diffuse distribution of brain cholinergic receptors, the use of these drugs is plagued by a great number of collateral effects. A certain degree of specificity can be conferred on this inhibitory effect by altering the physicochemical properties of the drug and these molecules can thus be excluded from the brain. However, when they are used to modify abnormalities of cholinergic receptor functions, the anticholinesterases exhibit a limited specificity.

In conclusion, the experience acquired on the indirect stimulation of synaptic receptors by inhibitors of the enzymes that inactivate the transmitter does not predict encouraging prospects for future drug developments.

2. Receptor inhibitors

Inhibition of postsynaptic receptors may lack an appropriate selectivity for reasons similar to those mentioned earlier in this report with regard to stimulation of postsynaptic receptors. Since the transmitter concentrations at receptors is a factor that controls the affinity constant of receptor blockers that act competitively, rates of transmitter release should be considered as a variable in the efficacy of these drugs. To assess this variable, the potency of these inhibitors should be tested in experimental conditions where the spontaneous rates of transmitter release from pertinent nerve terminals are different. During adaptation to cold exposure, the activity of certain central and peripheral catecholaminergic neurons is increased. This increase can be estimated *in vivo* by measuring the turnover rate of the transmitter involved. Using this approach, we have estimated NE and DM turnover rate in various tissues or brain regions of rats kept at two different environmental temperatures. The data of *Table 4* show that the turnover rate of heart and cerebellar NE is increased by cold exposure. From this finding one can infer that the central and peripheral catecholaminergic neurons do not respond to stressful situations with a diffuse and generalized increase of activity. Cold exposure may therefore be a suitable screening device to estimate the efficacy of postsynaptic receptor blockers in relation to various rates of neuronal activity.

Table 4. Turnover rate of dopamine (DM) and norepinephrine (NE) in various tissues of rats kept at 20°C and 4°C

Tissue	Catecholamine	nmole g^{-1}/± SEM		nmole g^{-1} h^{-1}	
		20°C	4°C	20°C	4°C
Heart	NE	5.2 ± 0.43	5.3 ± 0.48	0.78	2.2*
Salivary gland	NE	7.4 ± 0.58	7.8 ± 0.37	0.81	0.86
Stomach	NE	3.5 ± 0.27	3.2 ± 0.45	0.25	0.26
Cerebellum	NE	1.4 ± 0.093	1.2 ± 0.11	0.32	0.96*
Hypothalamus	NE	8.5 ± 0.82	8.7 ± 0.57	2.6	2.5
Striatum	DM	82 ± 6.3	94 ± 5.4	28	33

* *k* significantly different from that of rats kept at 20°C. Rats were kept at the indicated temperature for 14 hours before measuring turnover rate of NE by an isotopic method (pulse injection of 3,5-^{3}H tyrosine).

In vivo blockade of postsynaptic receptors is associated with an increased turnover rate of the transmitter stored in the nerve terminals facing the postsynaptic receptor that is blocked. This increase has been explained by several mechanisms: (a) collateral neuronal loops emanating from the cell inhibited by the blocker; (b) blockade of presynaptic receptors which are important to regulate transmitter release; (c) interference with reuptake mechanisms.

3. Postsynaptic receptors and second messenger as a site for drug action

Various schemes have been presented to depict how the message brought about by the action of the transmitter on receptors in the postsynaptic membrane is transferred to the pertinent intracellular compartments for further elaboration and transaction. Experiments performed with pineal gland[9], cervical sympathetic ganglia[10], and adrenal medulla[16] have clearly indicated that the stimulation of postsynaptic receptors may be linked with a change in the membrane-bound adenyl cyclase activity of postsynaptic cells. As first suggested by Sutherland and co-workers[17], the adenyl cyclase associated with postsynaptic receptors may be viewed as formed by a regulatory and catalytic unit; the transmitter would bind to the regulatory unit and by this binding alter the basic rate of intracellular formation of 3′,5′-cyclic adenosine monophosphate (cAMP). This nucleotide is believed to activate various enzymes intracellularly; since it mediates the effects of hormones and transmitter, it has been termed 'second messenger'. As an example of this interaction, we report in *Table 5* the effect of carbamylcholine injected into monolaterally splanchnicotomized rats on the cAMP concentration of normal and denervated adrenal medulla. These data show that this parasympathomimetic increases the concentration of cAMP in intact and splanchnicotomized medulla. Note the rapid rate of accumulation of cAMP at the time approach peak effect; this rate assures that an amount of cAMP equal to the steady state concentration of the nucleotide accumulates in the tissue in about one minute. Carbamylcholine, *in vitro*, does not reduce the activity of phosphodiesterase, the enzyme that catabolizes cAMP. Therefore, one must assume that under the stimulatory action of carbamyl-

choline, the turnover time of cAMP in adrenal medulla, it is even greater than that mentioned above because the nucleotide is continually metabolized while it accumulates in the medulla as a result of carbamylcholine action. Although the catabolic rate of medullary cAMP cannot be estimated precisely, it seems conceivable that it should be at least equal to the rate of cAMP accumulation elicited by aminophylline, a drug that inhibits phosphodiesterase. To estimate this rate, we have measured the cAMP accumulation in intact and splanchnicotomized adrenal medulla of rats receiving 200 μmoles kg^{-1} i.p. of aminophylline. These data are reported in *Table 5* and show that the rate of accumulation is greater in intact than in splanchnicotomized adrenal medulla. Since we have shown that aminophylline inhibits equally well the phosphodiesterase of normal and denervated adrenal medulla, we conclude that the afferent neurons to adrenal medulla regulate cAMP turnover rate by acting on adenyl cyclase.

Table 5. c-AMP concentrations in intact and splanchnicotomized medulla of rats receiving carbamylcholine (8.2 μmole kg^{-1} i.p.) or aminophylline (200 μmole kg^{-1} i.p.)

Minutes after injection	c-AMP p mole mg^{-1} protein (Mean ± SE)			
	After carbamylcholine		After aminophylline	
	Normal	Splanchnx	Normal	Splanchnx
0	33 ± 3.0	32 ± 2.3	42 ± 9	38 ± 4.6
3	30 ± 7.0	37 ± 8.0	88 ± 25	55 ± 12
6	53 ± 2.6	49 ± 5.6	190 ± 57	75 ± 20
12	200 ± 77	200 ± 67	420 ± 80	120 ± 75
24	450 ± 7.0	380 ± 54	380 ± 95	200 ± 60
48	110 ± 12	100 ± 19	500 ± 60	220 ± 40

Splanchnx was performed five days before the experiments. Each value is the mean of at least four experiments. The c-AMP was assayed by the luciferin–luciferase method

If, as will be shown later, cAMP plays a regulatory role in the elaboration of synaptic activity impinging upon the membrane of chromaffine cells, then one might change the response of the postsynaptic cells by injecting drugs that can modulate cAMP metabolism. An obvious site for this regulation is the phosphodiesterase. Uzunov and Weiss[11] reported that the soluble supernatant fraction of rat cerebellar homogenates contains various enzyme proteins with phosphodiesterase activity which can be separated by electrophoresis on a preparative polacrylamide gel column. Analyzing successive fractions of the column eluate for phosphodiesterase activity they found five distinct peaks of enzymatic activity designated I to V according to the order in which they emerged from the column. They also detected a discrete fraction containing a potent phosphodiesterase activator. This protein increases the activity of peak II about fourfold and that of peak V about two-fold, whereas the activities of peaks I, III, and IV were unaffected by the activator. Ca^{2+} increased the activity of peak II only. The five peaks

Table 6. Percentage inhibition of phosphodiesterases from rat cerebellum by various drugs

Drug	Type of phosphodiesterase*	Concentration				
		1×10^{-3} M	5×10^{-4} M	1×10^{-4} M	5×10^{-5} M	1×10^{-5} M
Chlordiazepoxide	I	0		0		0
	II	70		31		17
	III	74		26		0
	IV	28		24		0
Diazepam	I		0		0	
	II		69		26	
	III		59		35	
	IV		25		32	
Diphenylhydantoin	I					
	II		40			
	III		30			
	IV		20			
Aminophylline	I		0		0	
	II		80		20	
	III		40		0	
	IV		25		0	

* Classified according to Uzanov and Weiss

of phosphodiesterase also had markedly different stabilities, peaks III and V being the least stable ($t_{\frac{1}{2}}$ of 1 day when stored at 4°C).

In other studies, Uzunov and collaborators[12] established that in cell cultures on neuroblastoma, only peak III was present. Thus, the enzyme associated with peak III is of particular interest in screening neuropsychiatric drugs because it appears to reside in neurons. Since the adenylate cyclase system is ubiquitous and of physiological importance in various cells, the possibility of interfering selectively with neuronal phosphodiesterase appears to be an essential prerequisite for drug action in central nervous system. Drs. Strada and Suria have initiated such an exploration and their preliminary results are reported in *Table 6*.

The data reported in *Table 6* show that diazepam is slightly more effective than aminophylline in inhibiting the phosphodiesterase present in neurons (peak III). Moreover, if the activity of diazepam is compared with that of chlordiazepoxide, the former appears to be five-fold more active than the latter. Not only are these benzodiazepines structurally related to diphenylhydantoin, but they share its anticonvulsant action. The data reported in *Table 6* show that diphenylhydantoin is weaker than either benzodiazepine as a phosphodiesterase inhibitor. This finding may be relevant to explain the anticonvulsant activity of diphenylhydantoin and benzodiazepines because an action on the Purkinje cells of cerebellum appears to be important for their anticonvulsant effect[18,19]. It may be pertinent to note that NE injected microiontophoretically hyperpolarizes the Purkinje cells and this action appears to be mediated through cAMP[20]. It is also of interest that the phosphodiesterase inhibitory potencies of these three compounds rank in a sequence similar to their anticonvulsant activity.

4. Trans-synaptically controlled long-term regulation of neuronal function

Two main lines of evidence have contributed to our present understanding of the long-term regulation of tyrosine hydroxylase activity in the adrenal medulla. One was provided by Axelrod[21] by showing that tyrosine hydroxylase activity is regulated trans-synaptically, the other by Kvetnansky and collaborators[22] by showing that an injection of dibutyryl cyclic AMP restores the activity of tyrosine hydroxylase in hypophysectomized rats. Since afferent cholinergic nerves to the adrenal regulate medullary adenyl cyclase, we have investigated whether the prolonged increase of cyclic AMP in adrenal medulla elicited by aminophylline (*Table 5*), carbamylcholine (*Table 5*), and reserpine (*Table 7*) would be associated with a delayed increase of the tyrosine hydroxylase activity of adrenal medulla. To establish such a relationship, we have investigated whether drugs which promptly increase the concentration of medullary cAMP can after several hours increase the tyrosine hydroxylase activity of adrenal medulla. The results of these experiments are reported in *Table 7*.

These data show that the peak increase in cAMP concentrations is attained in about 12 minutes but the increase of tyrosine hydroxylase activity occurs 10 to 14 hours after the drug injection. The data reported in *Table 7* show that after injecting the various tyrosine hydroxylase inducers, accumulation of cAMP at its peak does not rank in parallel with the increase of tyrosine hydroxylase activity. However, the rate of cyclic AMP accumulation appears

related to the increase of tyrosine hydroxylase activity. We are presently investigating the sequence of biochemical events occurring in the time interval elapsing between accumulation of cAMP and the increase of tyrosine hydroxylase activity; we are now studying whether protein kinase activation and phosphorylation of acidic nuclear proteins and histones are among the events occurring in this time interval. In neurons, trans-synaptically mediated enzyme induction may be a physiological mechanism; accordingly, alteration of the trans-synaptic control of enzyme induction may cause neuronal pathology.

Table 7. Delayed increase of tyrosine hydroxylase activity and immediate accumulation of c-AMP in intact and denervated adrenal medulla of rats receiving various tyrosine hydroxylase inducers

Inducing drug	Denervation	Immediate changes in c-AMP†		Delayed changes in tyrosine hydroxylase activity as % of normal
		Extent of accumulation as % of normals	Accumulation rate (pmole mg^{-1} protein min^{-1})	
Reserpine	No	400	11	180*
(16 μmole kg^{-1} i.p.)	Yes	150	3	107
Carbamylcholine	No	1500	25	180*
	Yes	1100	25	150*
Aminophylline	No	1200	40	190*
(200 μmole kg^{-1} i.p.)	Yes	500	6	130

* $P < 0.05$

† The maximal accumulation of c-AMP is attained in about 12 minutes after drug injection. The accumulation rate of c-AMP is calculated from c-AMP concentrations measured between 6 and 12 minutes after the injection.

5. Methods to estimate drug effects on nerve terminal function

In *Table 1* I have listed several mechanisms located either on the nerve terminal membrane or intraneuronally, which are involved in the short- and long-term regulation of neuronal function. Some of these mechanisms can be easily tested *in vitro* in connection with drug screening. These include: (i) synthesis, (ii) catabolism, and (iii) uptake of transmitters.

(*i*) *Synthesis*

Transmitter biosynthesis is sometimes regulated through a feedback control by product inhibition of the rate-limiting enzyme for the transmitter biosynthesis. Actually, of the three best known putative transmitters, only the biosynthesis of catecholamines is regulated through a product inhibition of tyrosine hydroxylase activity[25]. The details of such a negative feedback are not thoroughly understood. It is known that catecholamines inhibit tyrosine hydroxylase by interfering with the pteridin cofactor[26], essential

for the function of this enzyme. Among the endogenous catechols, the highest affinity for this regulatory mechanism is displayed by DM. Since this NE precursor is free in the cytosol of sympathetic nerves where tyrosine hydroxylase is also located, DM may play a physiological role in the *in vivo* control of tyrosine hydroxylase[27]. If an excessive production of tyrosine hydroxylase were to be a recognized pathological cause of noradrenergic nerve disfunction, one may attempt to correct this defect by administering drugs that mimic the effect of DM or NE in the regulation of tyrosine hydroxylase activity, but not in their physiological action or in their binding properties to storage sites. Another approach to reduce excessive synthesis is to find inhibitors of pteridin reductase, the enzyme that maintains optimal concentrations of reduced pteridin cofactor[28].

The regulation for serotonin and acetylcholine biosynthesis seems to depend on the availability of substrate for the hydroxylating and acetylating enzyme, respectively. Thus within limits, serotonin synthesis depends on the concentrations of tryptophan available while acetylcholine synthesis is regulated by the availability of choline at the site of synthesis. However, the mechanisms involved are only apparently similar; in the case of acetylcholine, due to the excess of choline acetyltransferase, short-term regulation depends on the retrieval of choline by the nerve terminals[29]. Since this choline is formed from the recently-released acetylcholine, the rate of choline uptake regulates acetylcholine synthesis and relates to that of neuronal activity. Also, in the case of brain serotonin biosynthesis, the enzymes involved are not saturated by their respective substrates. Availability of substrates may also have a regulatory order. The regulation of the tryptophan availability at the sites of synthesis depends on the concentrations of freely-diffusable plasmatic tryptophan. Since free tryptophan is in equilibrium with tryptophan ionically bound to plasma protein, it follows that any molecule that competes with tryptophan for its binding sites in plasma might alter serotonin synthesis in brain. It remains to be ascertained whether the increase of 5-HT synthesis elicited by excesses of tryptophan availability has a functional significance.

The three types of regulation of transmitter biosynthesis described above are currently being studied in various laboratories; the outcome of such experiments may be of importance for future development of new drug screening procedures.

(*ii*) *Metabolism*

The existence of multiple forms of monoamine oxidase (MAO) has received strong support by a number of recent reports[30–32]. Johnston[31] has reported that clorgyline allows for the distinction of two forms of MAO in rat brain homogenates, an enzyme form termed Type A which is inhibited by low concentrations of clorgyline and an enzyme form designated as Type B which is relatively insensitive to these concentrations of clorgyline. The Type A enzyme is more effective than Type B enzyme in metabolizing serotonin while both enzymes are equally effective in metabolizing tyramine. Independent reports by Jarrot[33] and by Neff and Goridis[32] have shown that sympathetic nerves contain predominantly Type A MAO and that Type A

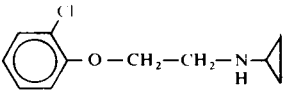
enzyme is very effective in metabolizing not only serotonin, but also norepinephrine and normetanephrine. Probably Type A enzyme is not a single entity; the ratio of tyramine to serotonin metabolized by Type A enzyme varies from tissue to tissue. Type B enzyme preferentially deaminates benzylamine. Tryptamine and dopamine, like tyramine, are metabolized equally well by both types of enzyme. This specificity of various forms of MAO toward certain substrates and their different sensitivity toward certain inhibitors opens new avenues for drug development in the field of MAO.

It is well known that the ingestion of foods containing high concentrations of biogenic amines by individuals receiving MAO inhibitors evokes episodes which resemble the clinical picture of pheochromocytoma (transient hypertension, headache, palpitation, nausea, vomiting, etc.). These symptoms are due to high blood levels of biogenic amines which are not detoxified by the

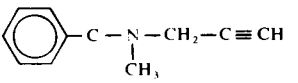
Table 8. In vitro properties of some MAO inhibitors*

Drug	MAO inhibition (K_I)			Vas deferens (Tyramine potentiation)
	Phenethylamine or benzylamine	Tyramine	Serotonin	
Lilly 51641 or clorgyline	600 (P)	300	1	(?)
Tranylcypromine	0.067 (P)	0.033	1	+
Pargyline	0.05 (P)	0.05	1	+
Deprenyl	0.01 (B)	?	1	Antagonism

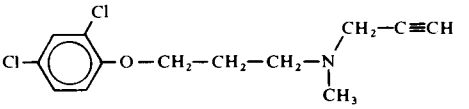
* Data from: J. Knoll and K. Magyar. *Adv. Biochem. Psychopharm.* 5, 393 (1972); R. W. Fuller, *Ibid.* 5, 339 (1972).



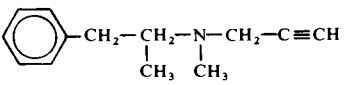
Lilly 51641



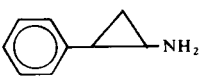
Pargyline



Clorgyline



Deprenyl



Tranylcypromine

liver enzyme. With the discovery of selective inhibitors of Type A enzymes, it appears that one might influence metabolism of serotonin and norepinephrine in monoaminergic nerves which contain Type A enzyme without altering the destruction by Type B enzyme of liver of the amines absorbed from the intestine. The data reported in *Table 8* show that the concentrations of clorgyline or Lilly 51641 required to block the metabolism of phenethylamine or tyramine are 600- and 300-fold greater than those required to inhibit 5-HT metabolism. In contrast, MAO inhibitors used clinically (pargyline and tranylcypromine) inhibit the metabolism of phenethylamine or tyramine better than that of 5-HT. Although the difference in affinity for various substrates displayed by Lilly 51641 may not yet be sufficient to obtain a satisfactory therapeutic index, it is possible that compounds with a satisfactory therapeutic index may be developed following such an approach.

Finally, I would like to draw attention to deprenyl, a MAO inhibitor developed by Knoll and Magyar[34] which fails to potentiate and may antagonize the biological effects of tyramine. They also obtained a very flat dose response curve for the inhibition of tyramine metabolism by this drug and a very prominent inhibitory activity toward benzylamine. This peculiar relationship suggests that the antienzymatic properties of deprenyl against various substrates should be studied more thoroughly because in the light of present developments, its weak inhibitory effect against tyramine should be considered an advantageous property.

(*iii*) *Uptake mechanisms*

Nerve terminals in the brain possess specialized uptake mechanisms for a variety of putative neurotransmitters, such as dopamine, norepinephrine, serotonin, choline, γ-aminobutyric acid, glutamic acid and glycine[35, 36]. Kinetic studies have elucidated optimal conditions for nerve endings to accumulate selectively their specific transmitter. By separating with density gradient centrifugation the synaptosomes (pinched-off nerve endings present in brain homogenates), populations of brain neurons relatively uniform with regard to the transmitter content can be separated and their biochemical properties studied. With regard to drug screening, it is important to consider that inhibition of monoamine uptake is a possible mechanism of action for various neuropharmacological agents. If a compound preferentially inhibits uptake of a transmitter, one usually infers that when this drug is injected into the animal, it will increase the time course of the transmitter–receptor interaction and will therefore prolong the duration of the receptor stimulation by the natural transmitter. Conceptually, this inference is acceptable, but it may not be practically verified because it implicitly assumes that a drug injected *in vivo* causes only one action. To verify whether blockade of uptake operates *in vivo*, one can integrate the results of *in vitro* measurements of uptake with turnover rate estimation of the transmitter *in vivo*.

6. Storage and release of transmitter as sites for drug action

Acetylcholine[37], catecholamine[38], and serotonin[39] are stored in nerve endings bound to synaptic vesicles. Nerve impulses release the transmitter by partial exocytosis[40], whereby only part of the vesicle content is ejected in the synaptic cleft. However, in this process not only the transmitter but

also enzyme proteins and other proteins contained in the synaptic vesicle reach the extracellular fluid[40]. Partial exocytosis brings the inside of each vesicle into contact with the extracellular fluid several times during its life cycle. Since during each exposure a proportion of the synaptic vesicles content is lost and since protein synthesis in nerve terminals is minimal and localized to the mitochondria, the participation of a given synaptic vesicle to successive partial exocytoses causes its reduction in size and its increase in electron density. Thus reduction in size expresses aging. This morphological counterpart of aging might explain the polymorphism exhibited by synaptic vesicles stored in a nerve terminal; the vesicles of small size and with dense core being the oldest members of the population of synaptic vesicles of a nerve terminal. Some authors have proposed that the smallest vesicles do not participate to exocytosis but still exchange their material with surrounding younger vesicles[40].

The various mechanisms involved in the storage process are not entirely understood; it is known that monoaminergic synaptic vesicles contain ATP and transmitter in a fixed ratio[41]. The storage process is sensitive to drug action, reserpine being the prototype of drugs that inhibit amine storage irreversibly[42]. In fact, in animals receiving reserpine, storage is back to normal only after the population of synaptic vesicles is completely renovated[43]. Although it is conceivable that reserpine binds covalently to some constituents of the synaptic vesicle, the molecular nature of the reserpine receptor is not known. Moreover, the reserpine receptor is not a generalized receptor present in all synaptic vesicles. Presumably, the vesicles that store GABA and acetylcholine are not affected by reserpine.

In practice there are simple neurochemical tests that characterize a reserpine-like action. These are: measurement of tissue monoamine concentrations; measurements of the brain concentrations of NE and 5-HT acidic metabolites; estimations of monoamines uptake with and without MAO inhibitors; estimatión of the uptake of an α-methyl-monoamine; assay of RNA concentrations in the cell body of monoaminergic neurons[44]; and measurement of the activity of enzymes involved in monoamine biosynthesis. During a reserpine-like action (a) the monoamine tissue concentrations are decreased for weeks; (b) in brain, the acidic metabolites of 5-HT and NE are increased because the amines are released intracellularly where they are metabolized by MAO; (c) the uptake of NE appears impaired but it is normalized if the animals are injected with MAO inhibitors; (d) the uptake of the α-amino analogue of NE is not impaired; (e) twenty-four hours after the injection of a reserpine-like drug, the RNA concentrations in tissues containing noradrenergic cell bodies (sympathetic ganglia) are increased; this increase reflects formation of synaptic vesicles; and (f) the activity of the enzymes involved in the monoamine biosynthesis is normal. From the foregoing, it appears that the long-lasting depletion of monoamines is insufficient to characterize a reserpine-like action. In fact. the injection of α-methyl monoamines (metaraminol, α-methylnorepinephrine, etc.) can cause a long-lasting depletion of peripheral catecholamines[45,46] similar to that elicited by reserpine. These drugs are taken up by nerve endings and stored in synaptic vesicles where they replace the transmitter[47]. They can be released by nerve impulses and are retrieved in nerve endings during the

resting phase. If the amine replacing the transmitter has an intrinsic activity lower than that of the natural transmitter, then, when it is released on to receptors, it causes a reduced response. For these reasons, such an amine is often termed a 'false neurochemical transmitter'. Two analogues of dopamine and 5-HT: 6-OH-dopamine and 6-OH-serotonin, respectively, concentrate in the two types of neurons. These molecules display a high chemical reactivity. When these two drugs accumulate in nerve terminals by virtue of their chemical reactivity, they cause permanent damage to the nerve endings[48]. Repairs to this damage can occur several months later by axonal sprouting. The depletion of tissue catecholamines caused by 6-OH-dopamine can be differentiated from that elicited by a false transmitter because the effect of 6-OH-DM is associated with a marked decrease of the enzyme's activities that are involved in the biosynthesis of NE. Some drugs are taken up by monoaminergic nerves (guanethidine is taken up by adrenergic nerves), but they cannot be readily stored in synaptic vesicles[44]. When guanethidine or some related compounds are injected in chronically high doses, they can cause destruction of the adrenergic nerves.

7. Drug effects on monoamine turnover rate

Figure 1 illustrates the present understanding of the processes involved in the release of catecholamines by nerve impulses. As shown in *Figure 1*, turnover rate of monoamines proceeds at a variable rate regulated by a feedback control operating at the level of the mixed function oxygenase. At rest, the amine uptake maintains the synaptic cleft free of transmitter and thus plays a role in regulating the function of nerve terminals (*Figure 1*). When the depolarizing wave reaches the nerve terminal, Ca^{2+} entry into the nerve terminal is facilitated. Simultaneously, by a phenomenon of partial

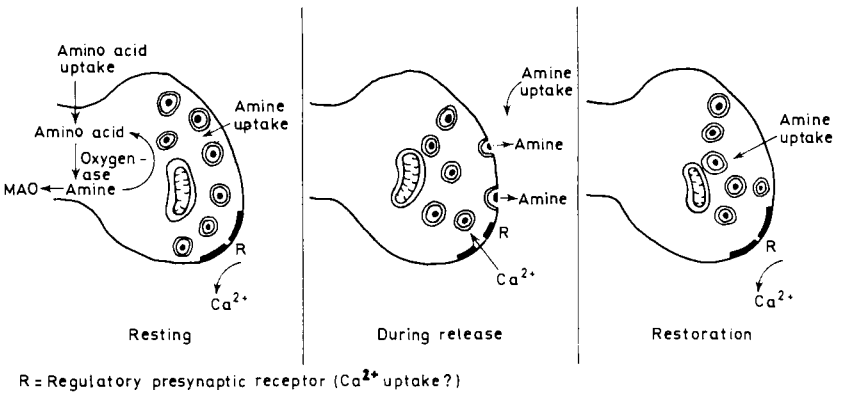


Figure 1. Speculative model of three phases of partial exocytosis. R is the presynaptic receptor that in this model is linked in an unknown way with the mechanism regulating Ca^{2+} uptake.

exocytosis, the amine is released from the synaptic vesicle into the synaptic cleft. At the time of maximal amine release by exocytosis the amine uptake is virutally nonexistent. This is also the time in which catecholamine concentrations in the synaptic cleft reach their peak. Perhaps by acting on a presynaptic regulatory receptor (R in *Figure 1*), the amine reduces the influx of Ca^{2+} and terminates the exocytosis which appears to be Ca^{2+}-dependent. Although our understanding of the molecular mechanisms involved and their sequence is at best fragmentary, we can hypothesize that drugs may facilitate or reduce the rate of transmitter efflux from nerve terminals by interacting with the various processes outlined in *Figure 1*. Drugs, by acting on the R receptor (*Figure 1*), may facilitate Ca^{2+} influx, thus prolonging the duration of exocytosis. Conversely, they may impair Ca^{2+} influx and reduce the transmitter release. The receptor R depicted in *Figure 1* is a regulatory site for the turnover rate of the transmitter which is susceptible to the action of drugs. These drug effects may be influenced by the nerve impulse activity. Therefore, drugs acting on the R receptor change the turnover rate of the transmitter to an extent which is related to nerve impulse activity. Other drugs may change the rate of transmitter turnover because they prevent the physiological retrieval of the released transmitter. With blockage of reuptake, interaction of the amine with the presynaptic R receptor is facilitated. As a result, the effect of these drugs tends to be independent of the rate of nerve activity. A summary of current views is presented in *Table 9*.

Table 9. Drug mechanisms affecting turnover rates of neuronal monoamines

Drugs increase turnover by:	Drugs decrease turnover by:
Blocking of amine uptake	Inhibition of the rate limiting enzyme
Blocking of postsynaptic receptors	Stimulation of postsynaptic receptors
Blocking of regulatory presynaptic receptors	Stimulation of regulatory presynaptic receptors
Lowering concentrations of the product functioning in feedback control	Increasing the concentrations of the product functioning in feedback control
Activating trans-synaptically the rate limiting enzyme	Increasing the rate of neuronal activity by an action on neuronal loops
Increasing the rate of neuronal activity by an action on neuronal loops	

8. Methods to measure monoamine turnover rate

Table 10 lists the methods that can be used to measure isotopically the turnover rate of 5-HT in brain and peripheral tissues. While the methods in (A) and (B) have been described in previous publications, method (C) was developed only recently[50]. In brief:

(i) DM concentrations in noradrenergic nerves can be measured by

Table 10. Isotopic methods to measure turnover rate of catecholamines and serotonin in brain and peripheral tissues

Tissue	Labelling procedure	Measurement	Calculation
(A) Peripheral tissues	L-^{3}H NE pulse injection	NE S.A.	$\frac{d[NE]}{dt} = -k_{NE}$ $k = a0.434$ a = slope of decline NE S.A.
(B) Brain or peripheral tissues	^{3}H tyrosine or ^{14}C tryptophan constant rate infusion	TY or TP S.A., NE, DM, 5-HT S.A.	NE S.A. $\frac{K}{k_T}\left[1 + \frac{1}{k_{NE} - k_T}\{k_T \exp(-k_{NE}t) - k_{NE}\exp(-k_T t)\}\right]$ K = rate of entry of label t = duration infusion k_T = fractional rate constant of tissue tyrosine
(C) Caudate, cerebellum, heart ventricles	^{3}H tyrosine pulse injection	DM and NE S.A. or Dopa and DM S.A.	$k_{DM}(t_1) = \frac{\Delta SA\ DM(t_1)}{\Delta t[SA_{Dopa} - SA_{DM}]t_1}$

multiple ion detection using gas chromatograph–mass spectrometry. To obtain gas chromatographic separation of DM, the perfluoropropionic derivative of the amine is obtained by reacting the amine with perfluoropropionic anhydride[51].

(ii) Dopa concentrations in caudate are measured fluorometrically as described in a previous publication[52].

Usually, we, as well as other authors, have measured turnover rate of 5-HT and NE from the change with time of the specific activity of the amino acid precursor at the rate-limiting step and that of the amine transmitter. Considering the model of DM biosynthesis in the caudate:

$$\underset{\text{(TY)}}{\text{Tyrosine}} \xrightarrow{k_{TY}} \text{Dopa} \xrightarrow{k_{Dopa}} \underset{\text{(DM)}}{\text{Dopamine}} \xrightarrow{k_{DM}}$$

The steady state equation 1 can be proposed

$$\frac{d[DM]}{dt} = k_{Dopa}[Dopa] - k_{DM}[DM] = 0 \tag{1}$$

If we have labeled the system with ^{3}H-TY, we can write equation 11

$$\frac{d[^3\text{H-DM}]}{dt} = k_{Dopa}[^3\text{H-Dopa}] - k_{DM}[^3\text{H-DM}] \tag{2}$$

by combining equation 1 and 2, we obtain equation 3

$$\frac{d\text{SA DM}}{dt} = k_{DM}(\text{SA Dopa} - \text{SA DM}) \tag{3}$$

where

$$\text{SA DM} = \frac{[^3\text{H-DM}]}{[DM]} \quad \text{and SA Dopa} = \frac{[^3\text{H-Dopa}]}{[Dopa]}$$

The question now arises whether we can substitute SA TY for SA Dopa. It is obvious that this substitution can be done if and only if SA TY = SA Dopa. This will be possible only if

$$\frac{d\text{ SA Dopa}}{dt} = 0$$

only in this particular case can we write (4)

$$\frac{d\text{ SA DM}}{dt} = k_{DM}(\text{SA TY} - \text{SA DM}) \tag{4}$$

Therefore equation 4, which is the equation frequently used to calculate turnover rate of DM[53] is valid only for a short and specific time interval following the injection of ^{3}H-TY. In practice, this implies that in order to determine the turnover rate of a transmitter we need data to plot a time curve of the specific activity of the transmitter itself and of the immediate precursor. However, it is also necessary to assess whether this precursor pool is totally metabolized to form the transmitter. The uniformity of the metabolic fate

of this immediate precursor can be ascertained by comparing the turnover rate of this precursor to that of the transmitter.

Table 11 lists the nonisotopic methods that can be used to measure turnover rate of brain 5-HT and tissue NE[53]. All are based on the assumption that the various agents used to perturb the steady-state (MAO inhibitors, α-methyltyrosine and probenecid) at the massive doses used in these experiments exert only one pharmacological action. This assumption is, however, rather unrealistic. Therefore, we must consider that these methods give, at best, only an indication of the turnover rate. In order to reduce the probability of error, it is advisable to estimate turnover rate using at least two methods in order to consolidate the indicative value of these methods.

Table 11. Nonisotopic methods to measure turnover rate of brain monoamines

Amine	Perturbation	Measurement	Calculation
NE	Blockade of tyrosine hydroxylase	Decline of NE	$\frac{d[NE]}{dt} = k_{NE}$ $k = a\ 0.434$ a = slope of NE decline
5-HT	Blockade of MAO	Accumulation of 5-HT	Accumulation of 5-HT
5-HT	Blockade of MAO	Decline of 5-hydroxy-indole acetic acid (5-HIAA)	$\frac{d[5\text{-HIAA}]}{dt} = k\ 5\text{-HIAA}$ $k = a\ 0.434$ a = slope of 5-HIAA decline
5-HT	Blockade of 5-HIAA transport by probenecid	Accumulation of 5-HIAA	Accumulation of 5-HIAA

9. Drug actions on brain monoamine turnover rate

(*i*) *Modifications of transmitter turnover rate unrelated to the rate of neuronal firing*

Although a change in turnover rate elicited by drugs can not be readily interpreted in terms of the molecular mechanism involved, measurement of turnover rates may still be useful to localize the possible anatomical site of drug action. Moreover, when drugs do not change steady state concentrations of the transmitter, estimates of transmitter turnover rate are of value for identifying the chemical nature of the transmitter involved in a given drug action. However, when one wishes to localize a pharmacological response to a certain brain area using as a criterion the change in transmitter turnover rate, one should ascertain that this relationship is valid for a number of doses. In other words, a relationship can be proposed only if the dose

response relationships between the two end points, pharmacological and turnover rate measurement, are similar. In *Table 12* I illustrate such a study for (+)-amphetamine, its stereo isomer, cocaine and aminorex. The data shown includes the most significant points of a dose response curve carried out with a wide dose range. It can be seen that for the drugs selected there is a remarkable correlation between the dose increasing motor activity and that accelerating the turnover rate of striatal DM. The turnover rate of telencephalic NE is not changed by the effective doses to increase motor activity. Hence, contrary to accepted opinion, *Table 12* shows that (+)-amphetamine and some chemically related compounds increase motor

Table 12. Effect of various drugs on motor activity and turnover rate of striatal DM and telencephalic NE

Drug	Events per min ± SE	Telencephalic NE (nmole g^{-1} h^{-1})	Striatal DM (nmole g^{-1} h^{-1})
Saline	4.5 ± 1.7	2.1 ± 0.31	38 ± 3.2
(+)-Amphetamine (1.4)	2.5 ± 0.71	1.7 ± 0.27	24 ± 2.9
(+)-Amphetamine (2.2)	25 ± 7.4*	2.3 ± 0.41	77 ± 7.2*
(−)-Amphetamine (7.4)	4.2 ± 1.8	1.9 ± 0.36	40 ± 3.8
(−)-Amphetamine (15)	22 ± 4.9*	2.7 ± 0.41	62 ± 6.4*
Cocaine (2.2)	6 ± 2.5	1.8 ± 0.33	35 ± 6.2
Cocaine (8.8)	22 ± 4.2*	1.7 ± 0.43	58 ± 3.8*
Aminorex (0.5)	7 ± 3.2	1.9 ± 0.43	41 ± 3.5
Aminorex (1.5)	77 ± 12*	2.2 ± 0.46	65 ± 3.2*

* $P < 0.05$

Rats (5 per group) received 1 mc kg^{-1} i.v. of 3.5-^{3}H tyrosine and 10 minutes later either saline or the drug listed in the *Table*; they were killed 15 minutes after the drug injection. Motor activity was measured during 15 minutes after drug injection. Steady state concentrations of NE and DM were not changed by the drugs during 15 minutes. At this time, the S.A. of tissue tyrosine was greater than that of either of the amines.

activity when given in doses that change neither the steady state concentrations nor turnover rate of telencephalic NE. The data show that doses of the drugs that increase motor activity also increase the turnover rate of striatal DM although they do not affect the steady state concentrations of this amine. This finding exemplifies the utility of turnover rate measurements to characterize the site of drug action.

(*ii*) *Modifications of transmitter turnover rates related to the rate of neuronal firing*

A characteristic property of the anticonvulsant diphenylhydantoin is that

of lowering post-tetanic potentiation (PTP) in a number of preparations. We have studied the action of diphenylhydantoin in the frog sympathetic ganglia. Since the concentration of diphenylhydantoin reducing PTP does not modify the response to single shocks, it may be inferred that diphenylhydantoin reduces the release of transmitter elicited by nerve impulses. Recent reports[18, 19] have indicated that the cerebellum is a site of action for diphenylhydantoin. This drug would increase the rate of firing of Purkinje cells which convey an important inhibitory input to the neurons they innervate. Stimulation of adrenergic cell bodies in *locus caeruleus* inhibits discharge rates of Purkinje cells[55]. The question then was: does diphenylhydantoin decrease this noradrenergic control because it prevents the release of transmitter from noradrenergic terminals? To answer this question, we studied the effect of diphenylhydantoin on the turnover rate of cerebellar NE in rats kept at 20°C and 4°C.

Table 13. Effect of diphenylhydantoin on the fractional rate constant of cerebellar NE in rats kept at 20°C and 4°C

Treatment (μmole kg^{-1} i.p.)	A 20°C $k\ h^{-1}$	B 4°C $k\ h^{-1}$	B/A
Saline	0.23	0.54	2.3
Diphenylhydantoin (196)	0.28	0.26	0.92
Diazepam (18)	0.28	0.44	1.5

k was measured from the rate of decline of NE in cerebellum of rats receiving 2 doses of α-methyltyrosine methyl ester.

The data shown in *Table 13* indicate that the fractional rate constant for cerebellar NE is increased by cold exposure and that this effect is completely prevented by diphenylhydantoin. Diazepam, an anticonvulsant chemically related to diphenylhydantoin which also reduces PTP in the frog sympathetic ganglia, seems to decrease the acceleration of cerebellar NE turnover rate elicited by cold exposure. One could speculate that by synergizing the effects of NE on the presynaptic regulatory receptor (*Figure 1*) both diazepam and diphenylhydantoin reduce postsynaptic potentiation and the turnover of NE in cerebellum. At this time, this is only a working hypothesis which I am presenting for your consideration as an example of the questions that can be asked when concepts of neurochemistry are applied to models for drug screening.

REFERENCES

[1] H. Ehringer and O. Hornykiewicz, *Klin. Wschr.* **38**, 1236 (1960).
[2] W. Birkmayer and O. Hornykiewicz, *Wien Klin. Wschr.* **73**, 787 (1961).
[3] H. Blaschko, *J. Physiol.* **96**, 50 (1939).
[4] A. Carlsson, *Pharmacol. Rev.* **11**, 490 (1959).
[5] C. A. Stone, C. A. Ross, H. C. Wenger, C. T. Ludden, J. A. Blessing, J. A. Totaro and C. C. Porter, *J. Pharmacol. Exp. Ther.* **136**, 80 (1962).

[6] S. Udenfriend and P. Zaltzman-Nirenberg, *J. Pharmacol. Exp. Ther.* **138**, 194 (1962).
[7] D. A. Walsh, E. G. Krebs, E. M. Reimann, M. A. Brostrom, J. D. Corbin, J. P. Hickenbottom, T. R. Soderling and J. P. Perkins, *Adv. Biochem. Psychopharmacol.* **3**, 265 (1970).
[8] G. A. Robison, R. W. Butcher, and E. W. Sutherland, *Ann. Rev. Biochem.* **37**, 149 (1968).
[9] B. Weiss and E. Costa, *Science* **156**, 1750 (1967).
[10] D. A. McAtee, M. Schorderet, and P. Greengard, *Science* **71**, 1156 (1971).
[11] P. Uzunov and B. Weiss, *Biochim. Biophys. Acta*, in press, 1972.
[12] P. Uzunov, H. M. Shein, and B. Weiss, in, *Proceedings of the Fifth Congress on Pharmacology*, Vol. 4, in press.
[13] N. H. Neff and E. Costa, in, *Proceedings of the International Symposium on Antidepressant Drugs*, p. 28. Excerpta Medica Foundation, New York (1967).
[14] G. C. Salmoiraghi, E. Costa, and F. E. Bloom, *Ann. Rev. Pharmacol.* **5**, 213 (1965).
[15] U. Ungerstedt, in, *6-Hydroxydopamine and Catecholamine Neurons.* (Ed. T. Malmfors and H. Thoenen), p. 101. Elsevier Pub. Co., New York (1971).
[16] E. Costa and A. Guidotti, in, *Proceedings of Symposium: Some New Concepts in Neurotransmitter Regulation.* San Diego, Calif., 1972, in press.
[17] G. A. Robison, M. J. Schmidt and E. W. Sutherland, *Adv. Biochem. Psychopharmacol.* **3**, 11 (1970).
[18] R. M. Julien and L. M. Holgen, *Life Sci.* **10**, 575 (1971).
[19] R. M. Julien, *Neuropharmacol.* **11**, 683 (1972).
[20] B. J. Hoffer, G. R. Siggins and F. E. Bloom, *Adv. Biochem. Psychopharmacol.* **3**, 349 (1970).
[21] J. Axelrod, *Science* **173**, 598 (1971).
[22] R. Kvetnansky, G. P. Gewirtz, V. K. Weise and I. J. Kopin, *Endocrinology* **89**, 50 (1971).
[23] J. Knoll and K. Magyar, *Adv. Biochem. Psychopharmacol.* **5**, 393 (1972).
[24] R. W. Fuller, *Adv. Biochem. Psychopharmacol.* **5**, 339 (1972).
[25] E. Costa and N. H. Neff, in, *Biochemistry and Pharmacology of Basal Ganglia.* (Ed. E. Costa, L. Cote, and M. D. Yahr), p. 141, Raven Press, New York (1966).
[26] T. Nagatsu, B. G. Levitt, and S. Udenfriend, *J. Biol. Chem.* **238**, 2910 (1964).
[27] E. Costa, *Advan. Biochem. Psychopharmacol.* **2**, 169 (1970).
[28] J. M. Musacchio, G. L. D'Angelo and C. A. McQueen, *Proc. Nat. Acad. Sci. USA* **68**, 2087 (1971).
[29] B. Collier and F. C. MacIntosh, *Can. J. Physiol. Pharmacol.* **47**, 127 (1969).
[30] M. B. H. Youdim, G. G. S. Collins and M. Sandler, *Nature* **223**, 626 (1969).
[31] J. P. Johnston, *Biochem. Pharmacol.* **17**, 1285 (1968).
[32] C. Goridis and N. H. Neff, *Brit. J. Pharmacol.* **43**, 814 (1971).
[33] B. J. Jarrott, *Neurochem.* **18**, 7 (1971).
[34] J. Knoll and K. Magyar, *Adv. Biochem. Psychopharmacol.* **5**, 393 (1972).
[35] S. H. Snyder, M. J. Kuhar, A. I. Green, J. T. Coyle and E. G. Shaskan, *Int. J. Neurobiol.* **13**, 127 (1970).
[36] M. J. Kuhar and S. H. Snyder, *J. Pharmacol. Exp. Ther.* **171**, 141 (1970).
[37] L. A. Barker, M. J. Dowdall, W. B. Essman, and V. P. Wittaker, in, *Drugs and Cholinergic Mechanisms in the CNS 193.* Research Inst. Nat. Defense, Stockholm, Sweden (1970).
[38] T. Hökfelt and A. Ljungdehl, *Adv. Biochem. Psychopharmacol.* **6**, 1 (1972).
[39] F. E. Bloom and E. Costa, *Adv. Cytopharmacol.* **1**, 379 (1971).
[40] A. D. Smith, *Pharmacol. Rev.* **24**, 435 (1972).
[41] L. B. Geffen and B. G. Livett, *Physiol. Rev.* **51**, 98 (1971).
[42] N. Weiner, G. Cloutier, R. Bjur and R. J. Pfeffer, *Pharmacol. Rev.* **24**, 203 (1972).
[43] A. Dahlström, *Phil. Trans. Roy. Soc. Lond. B* **261**, 326 (1971).
[44] J. Jarlestedt and A. Dahlström, *Neuropharmacology* **11**, 447 (1972).
[45] C. A. Stone and C. C. Porter, *Pharmacol. Dev.* **18**, 569 (1966).
[46] A. Carlsson, *Pharmacol. Rev.* **18**, 541 (1966).
[47] I. J. Kopin, J. E. Fischer, J. M. Maracchio, W. D. Horst and V. K. Weiss, *J. Pharmacol. Exp. Ther.* **147**, 186 (1965).
[48] F. E. Bloom, in, *6-Hydroxydopamine and Catecholamine Neurons.* (Ed. T. Malmfors and H. Thoenen) p. 135 Elsevier Pub. Co., New York (1971).
[49] C. C. Chang, E. Costa and B. B. Brodie, *J. Pharmacol.* **147**, 303 (1965).
[50] E. Costa, A. R. Green, S. H. Koslow, H. F. LeFevre, A. V. Revuelta and C. Wang, *Pharmacol. Rev.* **24**, 167 (1972).
[51] F. Karoum, F. Cattabeni and E. Costa, *Anal. Biochem.* **47**, 550 (1972).
[52] E. Costa, in, *Proceedings of the Fifth International Congress on Pharmacology*, Vol. 4, in press.

[53] N. H. Neff, P. F. Spano, A. Groppetti, C. T. Wang and E. Costa, *J. Pharmacol. Exp. Ther.* **176**, 701 (1971).
[54] E. Costa and N. H. Neff, in, *Handbook of Neurochemistry*, Vol. 4, p. 45. Plenum Press (1970).
[55] B. J. Hoffer, G. R. Siggins, P. A. Oliver and F. E. Bloom, *Ann. N. Y. Acad. Sci.* **185**, 531 (1971).

ACTIVE POLYPEPTIDES OF THE AMPHIBIAN SKIN AND THEIR SYNTHETIC ANALOGUES

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ABSTRACT

Amphibian skin represents an enormous store-house of biogenic amines and active peptides. So far four groups of peptides have been identified:

(a) *physalaemin-like peptides* (physalaemin, phyllomedusin and uperolein) possessing an intense action on vascular and extravascular smooth muscle as well as a potent action on lachrymal and salivary glands. Physalaemin is the most potent hypotensive agent so far described.

(b) *bradykinin-like peptides* (authentic bradykinin, phyllokinin, Val^1-Thr^5-bradykinin) displaying the well known effects on calibre and permeability of the capillaries.

(c) *caerulein-like peptides* (caerulein, phyllocaerulein) reproducing on the smooth muscle of the gall bladder and the gut, and on the exocrine secretions of the stomach, the pancreas, and the liver, all the actions of the intestinal hormone cholecystokinin-pancreozymin. Like this hormone, caerulein-like peptides also stimulate the secretion of insulin, glucagon and calcitonin.

(d) *bombesin-like peptides* (bombesin, alytesin, ranatensin) possessing a broad spectrum of activity on vascular and extravascular smooth muscle, on gastric acid secretion, and on the kidney, with potent activation of the renin–angiotensin system and stimulation of erythropoietin release.

With the exception of uperolein and phyllomedusin all the above peptides have been reproduced by synthesis together with a number of peptides or peptide fragments similar to the natural models. This has permitted some conclusions concerning the problem of structure–activity relationships.

Peptides of the amphibian skin are very similar or identical in their structure to active peptides occurring in mammalian tissues (substance P, bradykinins, cholecystokinin, gastrins). Hence their conspicuous interest, transcending the field of comparative pharmacology and biochemistry. It is possible that active amino acid sequences first discovered in amphibian skin may lead to the discovery of similar sequences, i.e. of new biochemical messengers, in mammalian tissues.

INTRODUCTION

The amphibian skin may be considered, as repeatedly stated[1], a store-house of biogenic amines and active polypeptides.

From the studies carried out by our research group during the past fifteen years on this exceptionally interesting and rich material some fundamental facts have emerged, which may be now regarded as firmly established.

An observation of substantial value is that all or nearly all amines and peptides found in amphibian skin have their counterpart in mammalian tissues, where they usually occur in a much lesser variety and concentration. Hence results obtained in the study of amphibian skin are of an interest transcending comparative pharmacology and biochemistry, as they may substantially contribute to the understanding and interpretation of facts assessed in mammals and may offer the basis for new research trends in higher vertebrates.

The active polypeptides so far detected in the amphibian skin may be divided into five groups characterized by distinctive features: physalaemin-like polypeptides or tachykinins, bradykinin-like polypeptides or bradykinins, caerulein-like polypeptides, bombesin-like polypeptides, and finally, miscellaneous polypeptides, a residual group in which those peptides are provisionally placed which still await elucidation of their structure or a sufficiently complete pharmacological study.

PHYSALAEMIN-LIKE PEPTIDES

At present, this polypeptide group is represented in the amphibian skin by the following three members:

(i) physalaemin, the prototype of the group, first isolated in a pure form from methanol extracts of the skin of *Physalaemus bigilonigerus* (*fuscumaculatus*) and present in skin extracts of other *Physalaemus* species as well (*Physalaemus centralis*, *Physalaemus bresslaui*). The content of physalaemin ranged between 370 and 700 μg per g dry skin[2, 3].

(ii) phyllomedusin, isolated from methanol extracts of the skin of the Amazonian hylid frog *Phyllomedusa bicolor* (1100 μg per g fresh skin)[4].

(iii) uperolein, found in the skin of Australian amphibians belonging to the genera *Uperoleia* and probably also *Taudactylus*. The elucidation of the structure of uperolein is in progress[5].

Two important physalaemin-like peptides occur outside the amphibian skin: eledoisin and substance P. The first is present, in large amounts (100 μg per g fresh tissue), in the posterior salivary glands of the Mediterranean octopod *Eledone moschata*[6]; the second is a polypeptide or, more likely, a family of polypeptides occurring in the brain and intestinal wall of vertebrates.

One substance P has been recently isolated from the bovine hypothalamus and, after elucidation of its structure[7], it has been reproduced by synthesis[8]. Formulae for these peptides are presented below.

Physalaemin	Pyr–Ala–Asp–Pro–Asn–Lys–Phe–Tyr–Gly–Leu–Met–NH_2
Phyllomedusin	Pyr———Asn–Pro–Asn–Arg–Phe–Ile–Gly–Leu–Met—NH_2
Eledoisin	Pyr–Pro–Ser–Lys–Asp–Ala–Phe–Ile–Gly–Leu–Met—NH_2
Substance P	Arg–Pro–Lys–Pro–Gln–Arg–Phe–Phe–Gly–Leu–Met–NH_2

From the amino acid sequences reported above it may be seen that all the four physalaemin-like peptides so far isolated in a pure form have in common the C-terminal tripeptide and the phenylalanine residue in position 5 from the C-terminus.

The promptness of their stimulant action on smooth muscle, has suggested the denomination of *tachykinins* for this polypeptide group, as opposed to the group of slow-acting kinins, the true bradykinins[1].

The tachykinins more thoroughly studied from a pharmacological point of view are eledoisin and physalaemin, which display the following fundamental actions:

(a) potent vasodilating and hypotensive action in most animal species, including man. The action is a direct one on the vascular smooth muscle. Threshold intravenous doses of physalaemin in the dog were of the order of 0.1 to 0.5 ng kg^{-1}, and a dose one million times greater could be tolerated by the animal with full recovery after 5 to 6 hours, which seems to be a unique example of tolerability. The polypeptide was very effective in antagonizing the pressor effects of catechol amines, nicotine and angiotensin in the dog. When given in the same quick intravenous injection 0.5 μg physalaemin completely abolished the hypertensive effect of 50–75 μg L-noradrenaline, 2 mg nicotine bitartrate and 10 μg angiotensin[9].

The vascular beds most sensitive to physalaemin and eledoisin in the dog were those of the hind limb musculature (minimal dose active by close intra-arterial injection < 1 pg), and the coronaries (threshold 10 pg)[10, 11]. Eledoisin infused locally into a branch of the left coronary artery of the dog at a rate of 8 ng kg^{-1} min^{-1} increased coronary sinus outflow by 20 per cent, coronary sinus oxygen tension by 10 per cent, and also increased stroke flow and cardiac oxygen consumption without affecting mean blood pressure and heart rate. Intravenous infusions of eledoisin were almost as effective in decreasing coronary vascular resistance, as were intracoronary infusions[12].

The blood vessels of the skin were less reactive than blood vessels in muscle. Renal and mesenteric vascular beds did not respond significantly to the tachykinins[10].

The following percentage changes in a number of cardiovascular parameters following intravenous injection of 4 ng kg^{-1} of physalaemin in the dog have been reported by Nakano, Darrow and McCurdy[13]: heart rate + 17.8, mean systemic arterial pressure −22.5, mean pulmonary arterial pressure +1·8, mean left atrial pressure −1, mean right atrial pressure +0.5, myocardial contractile force + 17.5, cardiac output + 52, total peripheral resistance −65.2, pulmonary vascular resistance −35.4.

Infusions of 0.6 μg min^{-1} of eledoisin in normal human subjects produced a transient fall in mean arterial blood pressure, tachycardia, marked increase in cardiac index, increase in stroke volume and fall in systemic vascular resistance. Blood flow to hand and forearm increased. More marked hypotension and tachycardia and a more pronounced increase in hand and forearm blood flows were observed during infusions of 2 to 5 μg min^{-1} of eledoisin. Intravenous infusions of 0.6 μg min^{-1} of eledoisin could be tolerated easily by all subjects studied, in spite of the occurrence of generalized intense erythema, burning of the eyes, throbbing in the head and dizziness. These results indicate that even in man eledoisin is a powerful dilator of vessels in skin and skeletal muscle and quite probably of vessels in other vascular areas[14].

(b) powerful stimulation of the salivary and lachrymal secretions in the rat, dog, man and hen by a direct effect on the secretory cells[15, 16]. Response

by the lachrymal glands could be obtained not only by systemic administration but also by instillation of the polypeptide into the conjunctival sac[17].

In the salivary glands of the dog physalaemin, besides stimulating the acinous secretory cells (threshold by intravenous injection 0.5 to 1.75 μg kg^{-1}), potently stimulated myoepithelial cells in the salivary ducts. Intravenous doses of physalaemin necessary to elicit a pressure rise in the submaxillary and parotid ducts of dogs were 100 times less than those active on salivary secretion[18].

Electrolyte and amylase concentrations in rat saliva which was evoked by physalaemin were virtually identical to those found in saliva evoked by stimulation of post-ganglionic nerve fibres[19].

The secretagogue effect of physalaemin in the dog pancreas was barely 1 per cent of that of caerulein[20].

(c) intense spasmogenic action on a number of isolated preparations of extravascular smooth muscle, among which the rabbit large intestine (threshold 0.2 to 1 ng ml^{-1}), the guinea-pig ileum (0.5 to 2 ng ml^{-1}), the human Fallopian tube (10 to 20 ng ml^{-1}), the rat urinary bladder (0.5 to 2 ng ml^{-1})[3, 21, 22].

On the *in situ* jejunal loops of the anaesthetized dog, physalaemin was twice as potent as cholecystokinin, on a molar basis, 15 times as potent as human gastrin I, 50 to 100 times as potent as either bradykinin or carbachol, and more than 300 times as potent as acetylcholine, eserine, histamine, vasopressin and 5-HT. Only caerulein overcame physalaemin in its stimulant effect, by three times[23].

Other extravascular smooth muscle preparations were poorly sensitive to the polypeptide: rat uterus, rat colon, dog, cat and rabbit urinary bladder[3, 22].

(d) positive action on capillary permeability in the guinea-pig, rat and man[24, 25]. By intradermal administration eledoisin caused in man pain, local oedema and erythema at doses above 1 ng. However, the polypeptide failed to elicit any pain response when injected intraperitoneally, subcutaneously or intramuscularly into human subjects at doses of 17 μg, 34 μg and 50 μg, respectively[26].

Eledoisin was removed from the circulation mainly by the kidney. The half-life of the polypeptide in the circulation was calculated to be less than 30 seconds. However it seemed that eledoisin was being bound to some constituents of the tissues, without actual destruction, and after the infusion of eledoisin stopped, it was being leached into the circulation again, thereby maintaining the blood levels for a longer time than expected[27].

Eledoisin has been reported to increase blood flow and lower peripheral resistance in a number of patients suffering from peripheral vascular diseases[28–31]. Physalaemin, in its turn, gave apparently satisfactory results in the treatment of the Sjögren syndrome and similar morbid conditions, characterized by a defect in lachrymal and salivary secretions[32].

The relative potency, on several preparations, of the natural tachykinins physalaemin, phyllomedusin and eledoisin is shown in *Table 1*.

It may be seen that whereas distinction, by parallel bioassay, of physalaemin from phyllomedusin was difficult or even virtually impossible (dog urinary bladder?), the distinction of physalaemin from eledoisin was rather

Table 1. The relative potency, on twelve preparations, of physalaemin, phyllomedusin and eledoisin (physalaemin = 100)

Test preparation	Phyllomedusin	Eledoisin
Dog blood pressure	40–70	25–30
Rat salivary secretion	100–200	not tested
Rabbit large intestine	60–150	30–75
Guinea-pig ileum	30–80	30–70
Rat duodenum	70–150	1000
Rat colon	100–150	1000–3000
Rabbit uterus	100–350	1000
Hamster urinary bladder	200	10000
Rat urinary bladder	80–120	30–100
Dog urinary bladder	600–800	5000
Monkey urinary bladder	100	3000–5000

easy, because some indexes of discrimination between the two peptides were very high[33].

More than 150 physalaemin- and eledoisin-like peptides have been synthesized in an attempt to elucidate the problem of the relationship between chemical structure and biological activity, and to dissociate the main pharmacological actions peculiar to the tachykinins[34–42].

Table 2 illustrates the relative biological activity of some physalaemin-like and eledoisin-like peptides. The activity of physalaemin was considered equal to 100 and that of the other compounds was expressed in terms of this activity.

From the data shown in the *Table* and from the more numerous data reported in the pertinent publications, the following conclusions may be drawn:

(i) By means of a progressive elimination of the N-terminal amino acid residue up to the C-terminal hexapeptide it was possible to reduce considerably the size of the physalaemin molecule, without consistently reducing the hypotensive action. The spasmogenic effect on the rabbit and guinea-pig intestine could even be conspicuously increased. A minimum of five amino acid residues was necessary in order to have an appreciable activity (0.3 to 1 per cent). The C-terminal hexapeptide of physalaemin had 50 per cent of the hypotensive action of the parent polypeptide, and approximately the same spasmogenic action on the guinea-pig ileum. However, the sialagogue activity was barely 3 per cent of that of physalaemin, and even the C-terminal octapeptide had only 30 per cent of the sialagogue activity of the parent endecapeptide[42].

(ii) The terminal amide group was apparently not essential for biological activity. In the C-terminal hexapeptide of eledoisin methionine nitrile could replace the methioninamide residue with no loss of hypotensive activity. However Met-NH_2 could not be replaced by Met-$N(CH_3)_2$.

(iii) Whereas methioninamide could not be replaced by other naturally occurring amino acids, it could be substituted, even with advantage, by non-natural synthetic sulphur-containing amino acids, such as ethioninamide and a variety of alkylhomocysteinamide residues.

Table 2. Relative potency of physalaemin-like peptides, on a weight basis (physalaemin = 100)

	Peptide	Test preparation: Dog blood pressure	Rabbit large intestine	Guinea-pig ileum
1	Pyr–Ala–Asp–Pro–Asn–Lys–Phe–Tyr–Gly–Leu–Met–NH_2 Physalaemin	100	100	100
2	Pyr–Pro–Ser–Lys–Asp–Ala–Phe–Ile–Gly–Leu–Met–NH_2 Eledoisin	22	60	50
3	Pyr——Asn–Pro–Ans–Arg–Phe–Ile–Gly-Leu–Met–NH_2 Phyllomedusin	40–70	60–150	30–80
4	Ala–Asp–Pro–Asn–Lys–Phe–Tyr–Gly–Leu–Met–NH_2	70	125–270	80
5	Asp–Pro–Asn–Lys–Phe–Tyr–Gly–Leu–Met–NH_2	35	60	70
6	Pro–Asn–Lys–Phe–Tyr–Gly–Leu–Met–NH_2	80–85	100	n.t.
7	Asn–Lys–Phe–Tyr–Gly–Leu–Met–NH_2	15	70	35
8	Lys–Phe–Tyr–Gly–Leu–Met–NH_2	50–85	500–750	130
9	Phe–Tyr–Gly–Leu–Met–NH_2	0.3	1.2	0.5
10	Orn–Phe–Tyr–Gly–Leu–Met–NH_2	45	400	80
11	His–Phe–Tyr–Gly–Leu–Met–NH_2	18	55	25
12	Arg–Phe–Tyr–Gly–Leu–Met–NH_2	45	n.t.	50
13	Ala–Phe–Tyr–Gly–Leu–Met–NH_2	5	50–100	20
14	Lys–Phe–Phe–Gly–Leu–Met–NH_2	50	n.t.	50
15	Lys–Phe–*m*-Tyr–Gly–Leu–Met–NH_2	45	900	105
16	Lys–Phe–Tyr(OMet)–Gly–Leu–Met–NH_2	55	250	130
17	Pyr–Ala–Asp–Pro–Asn–Lys–Phe–Ile–Gly–Leu–Met–NH_2	20	80	50
18	Ala–Asp–Pro–Asn–Lys–Phe–Ile–Gly–Leu–Met–NH_2	55	120	75
19	Pro–Asp–Lys–Phe–Ile–Gly–Leu–Met–NH_2	80	330	115
20	Lys–Phe–Ile–Gly–Leu–Eti–NH_2	45–90	380	155
21	Asp–Ala–Phe–Ile–Gly–Leu–Met–NH_2	6–7	35	18
22	Ala–Phe–Ile–Gly–Leu–Met–NH_2	3–4	18	7
23	Ser–Phe–Ile–Gly–Leu–Met–NH_2	3–4	33	12
24	Phe–Phe–Ile–Gly–Leu–Met–NH_2	4–5	35	30
25	Pro–Phe–Ile–Gly–Leu–Met–NH_2	10	35	10
26	Met–Phe–Ile–Gly–Leu–Met–NH_2	4–5	55	30
27	Ala–Trp–Ile–Gly–Leu–Met–NH_2	<0.5	2.5	1.5
28	Ala–Ala–Ile–Gly–Leu–Met–NH_2	<0.2	<0.2	<0.01
29	Ala–Phe–Ile–Ala–Leu–Met–NH_2	4.5	40	22
30	Ala–Phe–Ile–Phe–Leu–Met–NH_2	<0.1	<0.3	<0.25
31	Ala–Phe–Ile–Sar–Leu–Met–NH_2	5	35	28
32	Ala–Phe–Ile–Gly–Ile–Met–NH_2	<0.02	<0.1	<0.05
33	Ala–Phe–Ile–Gly–Ser–Met–NH_2	<0.04	<0.15	<0.05
34	Ala–Phe–Ile–Gly–Ala–Met–NH_2	<0.05	2	<0.05
35	Ala–Phe–Ile–Gly–Leu–(*S*-methyl)Hcys–NH_2	3	18	8
36	Ala–Phe–Ile–Gly–Leu–(*S*-ethyl)Hcys–NH_2	18	130	40
37	Ala–Phe–Ile–Gly–Leu–(*S*-propyl)Hcys–NH_2	20	180	30
38	Ala–Phe–Ile–Gly–Leu–(*S*-benzyl)Hcys–NH_2	5	65	40
39	Ala–Phe–Ile–Gly–Leu–(*S*-methyl)Cys–NH_2	<0.02	<0.1	<0.05
40	Ala–Phe–Ile–Gly–Leu–(*S*-ethyl)Cys–NH_2	<0.2	3	1
41	Ala–Phe–Ile–Gly–Leu–(*S*-benzyl)Cys–NH_2	<0.2	3	<0.5
42	Ala–Phe–Ile–Gly–Leu–Eti–NH_2	15–22	130	45
43	Lys–Phe–Ile–Gly–Leu–Eti–NH_2	45	360	80
44	Ala–Phe–Ile–Gly–Leu–Met–$N(CH_3)_2$	<0.05	<0.1	<0.05
45	Asp–Ala–Phe–Ile–Gly–Leu–Ala–NH_2	n.t.	n.t.	2

Table 2.—continued

	Peptide	Test preparation Dog blood pressure	Rabbit large intestine	Guinea-pig ileum
46	Asn–Ala–Phe–Ile–Gly–Leu–Ala–NH_2	n.t.	n.t.	1
47	Asn–Lys–Phe–Tyr–Gly–Leu–Gln–NH_2	n.t.	n.t.	0.3
48	BOC–Ala–Phe–Ile–Gly–Leu–Met–NH_2	5	110	80
49	BOC–Asn–Phe–Ile–Gly–Leu–Met–NH_2	3	65	25
50	(D)Asn–Phe–Ile–Gly–Leu–Met–NH_2	5	40	25
51	Ala–(D)Phe–Ile–Gly–Leu–Met–NH_2	<0.05	<0.1	<0.05
52	Ala–Phe–Ile–Gly–Leu–(D)Met–NH_2	<0.02	<0.1	<0.05

Eti, ethionine; Hcys, homocysteine; Pyr, pyroglutamic acid; *m*-Tyr, meta-tyrosine; Tyr(OMet) tyrosine methyl ether; BOC, *tert*-butyloxycarbonyl; n.t., not tested.

(iv) Substitution of the leucine or phenylalanine residues in the hexapeptide of either physalaemin or eledoisin produced a tremendous decay in the specific biological activity.

(v) Changes in biological activity produced by substitution of one of the three remaining amino acid residues were more irregular and apparently unpredictable. A high degree of activity was retained in hexapeptides where tyrosine (position 4 from the C-terminus) was replaced by valine, phenylalanine and isoleucine. However, hexapeptides in which tyrosine was replaced by leucine or alanine were practically devoid of activity. Likewise substitution of glycine (position 3) furnished in some cases active compounds, in other cases inactive compounds. Finally, lysine (position 6) could be replaced, with good preservation of activity, by a number of amino acids.

(vi) No significant changes in activity were noted when the N-terminal residue was protected by the *tert*-butyloxycarbonyl group.

(vii) The all D-enantiomer of the eledoisin hexapeptide was devoid of activity and did not antagonize the L-enantiomer of eledoisin. Similarly, replacement of an L-amino acid residue in the C-terminal pentapeptide with the corresponding D-amino acid residue caused a profound reduction of activity. However, the L-alanine in position 6 from the C-terminus could be substituted either by D-alanine or by D-asparagine with no important changes in activity.

(viii) Asn^5–eledoisin,Gly^5–Val^8–eledoisin and Asn^5–Val^8–eledoisin possessed 150, 80 and 60 per cent, respectively, of the activity of eledoisin.

It is evident that the results of these studies will gain a renewed attention after the elucidation of the structure of substance P which is no longer a humble polypeptide of the amphibian skin, but a noble constituent of the encephalic gray matter in all vertebrates, including man. All the above conclusions concerning relationship between chemical structure and biological activity are valid not only for the amphibian tachykinins but also for substance P.

BRADYKININ-LIKE PEPTIDES

In addition to authentic bradykinin (I), seven natural bradykinin-like polypeptides have been so far isolated in a pure form: kallidin or lysyl–bradykinin (II), methionyl–lysyl–bradykinin or methionyl–kallidin (III), Thr^6–bradykinin (IV), glycyl–bradykinin (V), polystes–kinin (VI), bradykinyl–isoleucyl–*O*-sulphate or phyllokinin (VII), and finally Val^1–Thr^6–bradykinin (VIII).

(I)	Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg
(II)	Lys–Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg
(III)	Met–Lys–Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg
(IV)	Arg–Pro–Pro–Gly–Phe–Thr–Pro–Phe–Arg
(V)	Gly–Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg
(VI)	Pyr–Thr–Asp–Lys–Lys–Leu–Arg–Gly–Bradykinin
(VII)	Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg–Ile–Tyr(SO_3H)
(VIII)	Val–Pro–Pro–Gly–Phe–Thr–Pro–Phe–Arg

Bradykinins (I), (II) and (III) occur in mammalian plasma, bradykinin (IV) in turtle plasma[43], bradykinins (V) and (VI) in the venom of *Polystes* wasps[44], and finally bradykinins (I), (VII) and (VIII) in the amphibian skin, together with the peptide Bradykinyl–Val–Ala–Pro–Ala–Ser (IX) which may be considered a bradykinin precursor.

Authentic bradykinin (I) has been isolated from the skin of the common European brown frog *Rana temporaria*, where it is present in amounts as high as 200 to 250 μg g^{-1} fresh tissue. Extracts of the skin of the European green frog *Rana esculenta* displayed a bradykinin-like activity corresponding to 10–25 μg bradykinin g^{-1} fresh skin[45].

Phyllokinin (VII) has been prepared in a pure form from skin extracts of the Brazilian frog *Phyllomedusa rohdei* and is probably present also in *Phyllomedusa bicolor* and in other *Phyllomedusa* species as well. It is the first and so far the only active natural bradykinin-like peptide with amino acid residues attached at the C-terminus of the bradykinin molecule. Trypsin digestion produced a splitting-off of the C-terminal dipeptide, giving rise to bradykinin which is known to be resistant to trypsin[46].

Desulphated phyllokinin, i.e. bradykinyl–isoleucyl–tyrosine, was consistently less active than phyllokinin on all tested preparations. The relative activity, on a molar basis, of phyllokinin and bradykinyl–isoleucyl–tyrosine in respect to that of bradykinin is shown in *Table 3*[47].

Val^1–Thr^6–bradykinin (VIII) has been isolated from extracts of the skin of the Japanese frog *Rana nigromaculosa*, together with authentic bradykinin and polypeptide (IX). Its stimulant activity on the rat uterus was about 20 per cent of that of bradykinin[48].

Since amphibian bradykinins display the same general activities as does bradykinin, these activities will not be discussed in detail. It will be sufficient to remember here some effects of the bradykinins which may be useful for their characterization and their distinction, in parallel bioassay, from other categories of active peptides.

The bradykinins are characterized by a remarkable but not exceptionally intense vasodilating and hypotensive action in the dog, rabbit and cat; by a

Table 3. The relative potency, on a molar basis, of bradykinin, phyllokinin and bradykinyl-isoleucyl-tyrosine (bradykinin = 100).

Test preparation	Phyllokinin	Bradykinyl-isoleucyl-tyrosine
Dog blood pressure	270–340	45–80
Rabbit blood pressure	40–90	15–30
Guinea-pig ileum	25–40	15–20
Rat uterus	30–40	20–25
Rat duodenum	7–9	n.t.
Human skin capillaries	130	n.t.
Rabbit urinary bladder	10–70	n.t.
Dog urinary bladder	40	n.t.
Cat urinary bladder	20	n.t.

n.t., not tested

potent stimulant action on the isolated guinea-pig ileum, cat small intestine and cat, dog and rabbit urinary bladder; and by a striking stimulant action on the oestrous uterus of the rat. They have a poor stimulant action on the rabbit and rat colon and display an inhibitory action on the rat duodenum. Bradykinin-like polypeptides effectively increase capillary permeability in man and experimental animals and cause pain when administered intra-arterially and intraperitoneally. All the known bradykinins are completely inactivated by incubation with chymotrypsin but are resistant to trypsin.

Bradykinins represent a polypeptide family having a widespread distribution in the amphibian skin. In fact bradykinin-like peptides occur, outside the species belonging to the genera *Phyllomedusa* and *Rana*, in species of other genera as well, among which are *Ascaphus truei*[49], *Physalaemus*, *Taudactylus* and *Litoria*.

Two recent localizations of kinin-like peptides outside the amphibian skin deserve particular mention. The first is that of bufokinin, present in methanol extracts of the urinary bladder of the toad *Bufo marinus paracnemis*[50], the second that of one or two bradykinin-like peptides in the skin of the lamprey *Eudontomyzon danforti vladykovi*[51].

Bufokinin is a negatively charged peptide, having a molecular weight of 1000 or slightly above, displaying the characteristic actions of bradykinin on isolated smooth muscle preparations and on blood pressure in the dog. Moreover, the peptide, like other bradykinins, when applied to the isolated toad bladder markedly inhibited the increase in the transepithelial osmotic water flux evoked by neurohypophysial hormones.

The lamprey in its turn contained in the skin small amounts of one or two biologically active bradykinin-like peptides. In contrast to authentic bradykinin they were inactivated not only by chymotrypsin but also by trypsin.

CAERULEIN-LIKE PEPTIDES

Caerulein is a decapeptide first isolated from methanol extracts of the skin of the Australian hylid frog *Litoria (Hyla) caerulea*, where it was present in concentrations of 100 to 1000 $\mu g g^{-1}$ fresh skin. The thick dorsal skin con-

tained 8 to 10 times more caerulein than the thinner ventral skin. Moderate losses of caerulein (20 to 40 per cent) occurred during drying of the skins[52, 53]. Authentic caerulein was later found in extracts of the skin of the South American leptodactylid frog *Leptodactylus pentadactylus labyrinthicus* and of the South African amphibian *Xenopus laevis* (300 to 800 μg g^{-1} fresh skin). That is of interest because *Xenopus laevis* may be easily bred in an aquarium[54]. It is probable that caerulein is present also in the skin of a number of other Australian hylid frogs (in *Litoria (Hyla)infrafrenata* and *Litoria (Hyla)moorei* up to 2500 to 3000 μg g^{-1} dry tissue), in other South American leptodactylid frogs (in *Leptodactylus laticeps* up to 1300 μg g^{-1} fresh tissue) and in other South African pipid frogs (in *Xenopus gilli* up to 1000 to 1500 μg g^{-1} fresh tissue)[53, 54].

The skin of the South American hylid frogs of the genus *Phyllomedusa* contained, in its turn, phyllocaerulein, a nonapeptide strictly related to caerulein. In *Phyllomedusa sauvagei*, whence it has been isolated in a pure form, it was present in amounts of 200 to 650 μg g^{-1} fresh skin. Similar amounts were present in the fresh skin of *Phyllomedusa bicolor*[55].

Systematic screening carried out in the last few years has shown that distribution of caerulein-like peptides in amphibian skin is broader than so far suspected. In fact, conspicuous amounts of caerulein-like peptides have been traced in *Nictimystes disrupta*, an amphibian of New Guinea, in *Hylambates maculatus* of South Africa and finally in some authentic frogs of New Guinea, Borneo and the Philippines, including *Rana erythraea*. The isolation of some of the above caerulein-like peptides is in progress. It is highly probable that the peptide in *Rana erythraea* is different from authentic caerulein[56–58].

The formulae below show the close chemical resemblance existing between the caeruleins, on the one side, and the gastrins and cholecystokinin–pancreozymin on the other side.

Pyr–Gln–Asp–Tyr(SO_3H)–Thr–Gly–Trp–Met–Asp–Phe–NH_2	Caerulein
Pyr–Glu———Tyr(SO_3H)–Thr–Gly–Trp–Met–Asp–Phe–NH_2	Phyllocaerulein
–Asp–Tyr(SO_3H)–Met–Gly–Trp–Met–Asp–Phe–NH_2	C-terminal octapeptide of cholecystokinin
–Tyr(SO_3H)———Gly–Trp–Met–Asp–Phe–NH_2	C-terminal hexapeptide of gastrin II

It may be seen that caerulein has in common with gastrin II the C-terminal pentapeptide and the sulphated tyrosyl residue, and with cholecystokinin the entire C-terminal octapeptide, with only the unimportant difference of a methionyl residue substituted for the threonyl residue at the 6-position from the C-terminus. It may be further noted that caerulein differs from phyllocaerulein only by the fact that the N-terminal tripeptide Pyr–Gln–Asp of the former is replaced in the latter by the dipeptide Pyr–Glu.

Caerulein has been subjected to extensive pharmacological investigation which showed that the polypeptide possessed an activity spectrum very similar to that of cholecystokinin–pancreozymin, together with conspicuous gastrin-like effects. The main pharmacological effects of caerulein may be summarized as follows:

(a) Potent spasmogenic action on the *in vivo* and *in vitro* gall bladder

musculature of all the tested animals, including man. A few nanograms per kilogram of weight injected intravenously were sufficient to stimulate the organ *in situ* and less than 1 ng kg^{-1} min^{-1} was effective when infused intravenously. The isolated gall bladder was contracted by caerulein in concentrations as low as 0.01 to 2 ng ml^{-1} of nutrient solution. There was no tachyphylaxis and, generally, a good dose–response relationship. The spasmogenic action of the polypeptide was atropine resistant[59].

In the dog the threshold cholecystokinetic doses by intravenous and subcutaneous routes were 1 and 10 ng kg^{-1}, respectively. The gall bladder contraction began very soon and lasted 3 to 4 hours after intravenous administration and 6 to 7 hours after subcutaneous administration. In some cases a certain degree of contraction was appreciable up to 24 hours. The cholecystokinetic activity was more striking after subcutaneous than after intravenous administration, except for threshold doses. Also the intrahepatic and extrahepatic bile ducts were apparently contracted by caerulein. On a molar basis caerulein was 16 times as potent as cholecystokinin and 170 times as potent as both gastrin I and gastrin II[60, 61].

In man caerulein has been largely used in cholecystography and cholangiography. Doses were 5 to 30 ng kg^{-1} by the intravenous route, 0.25 to 1 μg kg^{-1} by intramuscular or subcutaneous injection, and 0.75 to 1 μg kg^{-1} by nasal insufflation. The threshold intravenous dose was of the order of 1 ng kg^{-1}. The spasmogenic action of the polypeptide began soon after the injection, reached its peak after 10 to 15 minutes, and disappeared after 90 minutes; 2·5 to 5 ng kg^{-1} of intravenous caerulein produced a response very similar to that caused by a fatty meal[62–64].

(b) Relaxing action on the choledocho–duodenal junction, which was particularly evident when the tone of the sphincter was elevated, either spontaneously or following premedication with spasmogenic drugs[65]. As a consequence of the relaxation of the sphincter of Oddi the choledochal resistance was lowered and bile flow increased in conscious dogs. Of the peptides examined the most potent relaxant was caerulein[66].

(c) Powerful stimulant action on the *in situ* musculature of the gut, with the possible exception of the duodenum, at least in man.

In the intact, conscious dog, caerulein caused emesis and evacuation of the bowel. The mean effective dose by rapid intravenous injection was 0.4 to 0.5 μg kg^{-1}, and by subcutaneous administration 3 to 4 μg kg^{-1}. By intravenous infusion caerulein produced retching in most dogs when doses exceeded 10 ng kg^{-1} min^{-1}. Intravenous doses as low as 1 to 5 ng kg^{-1} had a spasmogenic action on jejunal loops of the dog, and slightly larger doses contracted the small intestine of the cat. The stomach and the large intestine were less sensitive to the polypeptide. Caerulein also displayed a considerable spasmogenic action on the rat pylorus. All these effects, with the exception of the latter, could be reduced or abolished by atropine[59].

In human subjects studied by the balloon method caerulein caused inhibition of the duodenal motility and stimulation of the jejunal motility and tone. Threshold doses by intravenous infusion were of the order of 1 ng kg^{-1} min^{-1}. The effect subsided a few minutes after the infusion had been discontinued. By the subcutaneous route the threshold dose was 25 to 30 ng kg^{-1}. With 0.75 μg kg^{-1} the stimulant effect lasted 30 to 40 minutes[67].

In small bowel contrast studies it could be seen, by fluorography and cinematography, that caerulein administration (1 to 2 ng kg^{-1} min^{-1} by infusion or 0.5 to 1 μg kg^{-1} by intramuscular injection) produced a conspicuous reduction in the transit time of the contrast medium. The barium took only 20 to 30 minutes to reach the colon and peristalsis was very lively. Not infrequently the contrast medium was present simultaneously in the colon and the stomach[68].

Some isolated preparations of intestinal smooth muscle were extremely sensitive to caerulein. They were used in the study of the mechanism of action of caerulein, which appeared to be different depending on the animal species and the intestinal section considered. In most cases it was predominantly cholinergic, in other instances it seemed a direct one[69, 70].

In the guinea-pig ileum caerulein has been shown to act on non-nicotinic receptors in the intramural ganglia causing a conspicuous release of acetylcholine. A similar release of acetylcholine occurred also in the rabbit duodenum[71].

Because of its actions on the intestinal smooth muscle, caerulein has been employed, apparently with success, in the treatment of postoperative gastrointestinal atony and other forms of adynamic ileus[72].

(d) Potent stimulant action on the exocrine pancreas causing the secretion of abundant pancreatic juice rich in enzymes. In the anaesthetized dog with an acutely cannulated Wirsung duct, threshold doses of caerulein were 1 to 5 ng kg^{-1} by rapid intravenous injection, 0.25 to 1 ng kg^{-1} min^{-1} by intravenous infusion, and 50 to 100 ng kg^{-1} by subcutaneous injection. An intense, constant flow of pancreatic juice could be maintained by infusion of caerulein up to 10 to 20 hours[73]. In conscious dogs provided with chronic pancreatic fistulas the dose of caerulein required for 50 per cent of maximal response was 0.5 ng kg^{-1} min^{-1}, volume output, and 0.7 ng kg^{-1} min^{-1}, enzyme output. Depending on experimental conditions, caerulein was 7 to 50 times more potent than gastrin, and 3 to 6 times more potent than cholecystokinin on pancreatic juice flow and enzyme output. Secretin, however, stimulated flow 2.5 to 20 times more than caerulein, on a molar basis[74, 75].

Repeated subcutaneous administration of caerulein caused a remarkable increase (up to 100 per cent) of amylase and chymotrypsin concentration in the rat pancreas[76] and a 80 to 100 per cent increase in the incorporation of ^{14}C–leucine into protein by pancreas tissue slices of fasted guinea-pigs[77]. In chickens the threshold dose of caerulein active on pancreatic secretion was 0.1 to 0.3 ng kg^{-1} min^{-1}. Maximal observed increases were 8-fold for volume output, 23-fold for amylase output, 18-fold for lipase output, and 28-fold for output of total tryptic activity. The concentration of enzymes in caerulein juice was 3- to 6-fold the concentration in control juice[78].

The pancreatic islets, too, were stimulated by doses of caerulein of the same order of magnitude as those active on the exocrine pancreas. In fact, in the dog, doses of the polypeptide as low as 2 ng kg^{-1} min^{-1} produced a 2.5- to 4-fold increase of the immunoreactive insulin levels in pancreatico-duodenal venous blood and a 3.5-fold increase of the immunoreactive glucagon levels. The effect lasted as long as the infusion was continued. Discontinuing the infusion caused a prompt return to basal values; recommencing the infusion resulted in a renewed release of insulin[79, 80].

In normal human subjects 10 ng kg^{-1} of caerulein administered by rapid intravenous injection produced a slight increase in blood glucose levels, while plasma immunoreactive insulin did not change. However, in some patients with insuloma, caerulein provoked a powerful insulin response[81].

Concomitantly with, and probably resulting from, the stimulation of the pancreas there was an increase in the blood flow through the duodenal–pancreatic artery. Intravenous doses of caerulein active on this vascular area (1 to 2 ng kg^{-1}) were at least 10 times lower than those causing systemic hypotension[82].

(e) Conspicuous stimulant action on the Brunner glands. The intravenous infusion of 2.5 ng kg^{-1} min^{-1} of caerulein stimulated the glands of the dog to produce 0.56 ml of secretion per 15 minutes, and the glands of the cat to produce 0.36 ml of secretion per 60 minutes[83].

(f) Potent stimulant action on gastric secretion, with increase in volume, acid and pepsin outputs. Concentration of hydrochloric acid in the dog juice increased up to 50 per cent, and concentration of pepsin up to 250 per cent[84].

In dogs with denervated gastric pouches the dose of caerulein needed to produce one-half of the maximal acid secretion was 8 ng kg^{-1} min^{-1}; in gastric fistula dogs 2.7 ng kg^{-1}. On both a molar and a weight basis caerulein has been found to be more potent than gastrin in stimulating acid secretion. However, it should be pointed out that gastrin was capable of producing much higher observed and calculated maximal responses than was caerulein. Calculated and observed acid outputs to maximal doses of gastrin were usually twice those found for caerulein. Caerulein was then extremely potent in that low doses produced significant amounts of secretion, but it was not effective in producing high rates of acid secretion comparable to those seen with maximal doses of gastrin or histamine[85, 86].

In man the threshold intramuscular dose of caerulein was 50 to 100 ng kg^{-1} and the optimum response was obtained with 250 ng kg^{-1}. At this dosage the effect lasted 60 to 90 minutes and peak gastric secretion (40 ml juice and 3.3 m equiv total HCl output) was reached between 15 and 30 minutes. During the course of the response to 250 ng kg^{-1} of caerulein, 35 m equiv Cl^-, 10 m equiv Na^+, and 5 m equiv K^+ were secreted[86, 87].

Gastrin- and pentagastrin-induced gastric secretion was inhibited by caerulein; the effect of the polypeptide on histamine-induced secretion was, on the contrary, variable. In its turn, the effect of caerulein was completely abolished by atropine in the dog, man and chicken, but was atropine-resistant in the rat and the pigeon[84, 87, 88].

Caerulein increased 'short circuit current' and simultaneously secretion of hydrochloric acid in the isolated gastric mucosa, starting from concentrations as low as 10^{-10} M. The effect was atropine-resistant. On this preparation caerulein was 10 000 times more active, on a molar basis, than either pentagastrin and human gastrin I, 30 times as active as cholecystokinin and 1000 times as active as histamine[90].

In the rat, caerulein caused also a remarkable increase in the secretion of the intrinsic factor. With 0.5 μg kg^{-1} of the polypeptide given subcutaneously the increase in intrinsic factor secretion was 100 per cent; with 5 μg kg^{-1}, 350 per cent. By intravenous infusion the threshold dose of caerulein was

10 ng kg^{-1} min^{-1}. On a molar basis caerulein was more than 10000 times as active as histamine[91].

Finally, caerulein always produced an increase in the histidine decarboxylase activity of the gastric mucosa in the rat. The threshold dose for a three-hour infusion period appeared to be 0.5 μg kg^{-1} h^{-1}. With 5 μg kg^{-1} h^{-1} the enzyme activity was increased by 400 per cent[92].

(g) Variable effects, depending on the animal species considered, on flow and composition of hepatic bile, as well as on transhepatic transport of bile salts, bile pigments and organic anions[93–97].

In the chicken, which was the species most thoroughly studied up to the present, the intravenous infusion of caerulein elicited the following effects: (*i*) increase in the volume of bile flow. The threshold dose of the polypeptide was 0.5 ng kg^{-1} min^{-1} and maximum increase in bile flow was 8-fold; (*ii*) increase in output of bile salts, cholesterol, pigments, and bicarbonate. At an infusion rate of 0.5 ng kg^{-1} min^{-1} the output of bile salts increased by 25 per cent and that of cholesterol by 75 per cent. Except for bicarbonate, the concentration of the bile components was usually higher in the caerulein bile than in control bile; (*iii*) increase in the excretion rate of exogenous bile salts, with removal of the autoinhibition produced by infusion of large amounts of these salts. With 15 ng kg^{-1} min^{-1} increase was 5-fold; (*iv*) acceleration of the transhepatic transport of sulphobromophthalein with simultaneous increase in plasma BSP clearance (threshold 1 ng kg^{-1} min^{-1} caerulein); (*v*) acceleration of the transhepatic transport of rose Bengal and indocyanine, with simultaneous increase in the secretion of endogenous bile salts[93].

Table 4. The effect of caerulein on bile flow and bile salts secretion in different animal species

Animal species	Bile flow	Secretion of bile salts	
		Total output	Concentration
Chicken	+++	++	+, 0
Goose	+++	++	0
Pigeon	+	+++	+++
Rabbit	+	++	+++
Cat	++	++	++
Dog	++	+	(?)
Rat	(+)	0	(−)
Guinea pig	0	0	0

0, no effect; +, increased; −, reduced

In the dog, half of the maximal calculated increase in bile flow was produced by 0.25 ng kg^{-1} min^{-1} of caerulein[94]. At variance with previous findings, not only the output of bicarbonate and chloride but also that of bile salts was increased following the administration of the polypeptide.

A synopsis of the actions produced by caerulein on biliary secretion in different animal species is presented in *Table 4*. Results have been obtained in animals with their gall bladder excluded, with total diversion of bile for collection and with continuous administration of bile salts to support bile flow[97].

It may be seen that the rat and guinea-pig were totally, or nearly, unresponsive to caerulein. In the goose the powerful stimulation of bile flow was not accompanied by any increase in the concentration of bile salts, whereas in the rabbit, cat and pigeon bile secreted under the influence of caerulein showed a high concentration of bile salts.

Of considerable interest are the findings that caerulein (10 to 20 ng kg^{-1} min^{-1}) produced also in the rabbit an increase in the maximal rate of secretion of sulphobromophthalein and bilirubin into the bile and that in the chicken, rabbit and pigeon the choleretic effect of caerulein was generally magnified if the animal was supplied with exogenous bile salts via the duodenum instead of intravenously.

More work is necessary to elucidate the mechanism of the salteretic effect of caerulein. It is certain that the polypeptide enhances the transhepatic transport of bile salts and hence their canalicular excretion; it may also be that it acts on intestinal reabsorption of bile salts and that it affects 7-α-hydroxylation of cholesterol, which is the rate-limiting step in the biosynthesis of bile acids.

(h) Conspicuous villokinetic activity in the cat and in birds. The intravenous infusion of caerulein or cholecystokinin in animals with or without drainage of digestive secretions stimulated the pump-like movements of chicken, cat and pigeon duodenal–jejunal villi. Threshold infusion rates for caerulein were 0.25, 0.5 and 2 ng kg^{-1} min^{-1}, respectively. Dose–response relationship was usually modest, and an inhibitory action could supervene with supramaximal rates in the chicken and cat, but not in the pigeon[98,99].

(i) Moderate action on the systemic blood pressure. In dogs and rabbits caerulein nearly always produced hypotension with a good dose–response relationship. In the dog the threshold dose was 10 to 100 ng kg^{-1} by intravenous injection, 5 to 15 ng kg^{-1} min^{-1} by intravenous infusion, and 5 to 10 μg kg^{-1} by subcutaneous injection. In other animal species blood pressure response was more erratic and unpredictable[100].

(j) Potent stimulant effect on calcitonin release from porcine thyroid, perfused *in situ*. Approximately 1.3 ng ml^{-1} of caerulein doubled the secretion rate of calcitonin. On a molar basis the polypeptide was about twice as active as cholecystokinin[101].

The acute toxicity of caerulein in animals seems to be very low: in the mouse LD50 by intravenous injection was 1030 mg kg^{-1}.

Very high doses of caerulein (50 and 250 μg kg^{-1}) given daily to rats by subcutaneous injection, for periods of 1, 3 and 6 months, produced in the pancreas severe acinar cell damage ending in parenchymatous atrophy with diffuse fibrosis and increase of ductular and centroacinous cells. The endocrine pancreas, however, was not damaged and the above severe lesions of the exocrine tissue did heal spontaneously after discontinuing the injections of caerulein. Pancreatic lesions were accompanied by an impairment of fat absorption, evidently due to lack of pancreatic lipases, and by reduction of fat deposits. In addition to the pancreatic changes caerulein produced also hypertrophy of the duodenal wall and of the gastric parietal cells, events which could suggest a trophic action of the polypeptide.

Daily doses of 10 μg kg^{-1} caerulein, administered subcutaneously up to 6

months, resulted only in hypertrophy of the pancreas and no pathological changes.

No foetal malformations were observed in rats and rabbits given caerulein during the whole pregnancy at daily subcutaneous doses of 50 and 25 μg kg^{-1}, respectively[102, 103].

Because caerulein is now very often studied and discussed together with cholecystokinin and the gastrins it seems useful to condense in *Table 5* available data on the relative potency of the three peptides on a number of test preparations. The activity of caerulein was considered equal to 100 and that of cholecystokinin and human gastrin I was expressed in terms of this activity.

Table 5. Relative potency, on a molar basis, of caerulein, cholecystokinin and human gastrin I (caerulein = 100)

Test preparation	Cholecystokinin	Gastrin I
Gall bladder *in situ*		
man	6–7	0.6
guinea-pig	30–35	n.t.
dog	10	0.25
Dog small intestine *in vivo*	17	2–2.5
Choledocho-duodenal junction (guinea-pig)	5	n.t.
Intestinal villi (chicken)	50	n.t.
Dog systemic blood pressure	13–25	2–3
Duodenal–pancreatic artery (dog)	10–15	n.t.
Perfused rat stomach preparation	35	5–20
Isolated frog gastric mucosa	3	0.01
Dog exocrine pancreas	15–30	2–15
Dog Brunner glands	10	10

n.t., not tested.

Approximately 75 caerulein-like polypeptides have been synthesized by our research group in order to contribute to the elucidation of the problem of structure–activity relationship for caerulein and cholecystokinin and in an attempt to dissociate the different actions of caerulein[104, 105].

The relative activity of a number of synthetic peptides related to caerulein is shown in *Table 6*, in which the activity of a given weight of caerulein was considered equal to 100 and the activity of the same weight of the other peptides was expressed in per cent.

From the tabulated data and from other published and unpublished results, the following conclusions may be drawn:

(i) The whole activity spectrum of caerulein was present in the C-terminal heptapeptide, and further lengthening of the peptide chain was of limited effect.

(ii) A necessary prerequisite for the activity of the heptapeptide was the presence at its N-terminus of an *O*-sulphated tyrosyl residue or another appropriate negatively charged residue. Thus, desulphation of the tyrosyl residue or its replacement by a *p*-sulphonamido–phenylalanyl residue produced a drastic decay of activity. Substitution of the sulphuric acid by

Table 6. Relative potency of caerulein-like peptides, on a weight basis caerulein = 100)

	Peptide	Test preparation: Denervated gastric pouch of the dog	Dog pancreas	Guinea-pig gall bladder
1	Pyr–Gln–Asp–Tyr(S)–Thr–Gly–Trp–Met–Asp–Phe–NH_2 Caerulein	100	100	100
2	Pyr–Glu–Tyr(S)–Thr–Gly–Trp–Met–Asp–Phe–NH_2 Phyllocaerulein	110–150	110–150	120–150
3	Pyr–Gln–Asp–Tyr–Thr–Gly–Trp–Met–Asp–Phe–NH_2	2.5	4	< 1
4	Pyr–Asp–Tyr(S)–Thr–Gly–Trp–Met–Asp–Phe–NH_2	40	50	40–60
5	Tyr(S)–Ala–Ala–Gly–Trp–Met–Asp–Phe–NH_2	3	1.4–4	< 1
6	Tyr–Thr–Gly–Trp–Met–Asp–Phe–NH_2	< 1	1.2–2.5	0.05
7	Tyr(S)–Thr–Gly–Trp–Met–Asp–Phe–NH_2	20–40	60–80	30–50
8	Tyr(P)–Thr–Gly–Trp–Met–Asp–Phe–NH_2	5·5–7	5–10	< 1
9	Tyr–Gly–Trp–Met–Asp–Phe–NH_2	0.5–1	1.5–3	< 0.1
10	Tyr(S)–Gly–Trp–Met–Asp–Phe–NH_2	8–20	10–30	< 0.5
11	Tyr(P)–Gly–Trp–Met–Asp–Phe–NH_2	2	5–7	< 1
12	Tyr(S)–Thr–Trp–Met–Asp–Phe–NH_2	2–3	3–5	10–15
13	Tyr(S)–Trp–Met–Asp–Phe–NH_2	2–3	1–2	< 1
14	Tyr(P)–Trp–Met–Asp–Phe–NH_2	2–3	2–3	< 1
15	Tyr(S)–Gly–Gly–Trp–Met–Asp–Phe–NH_2	3–4	10–15	4–10
16	Tyr(S)–Met–Gly–Trp–Met–Asp–Phe–NH_2	n.t.	65–70	80–150
17	Tyr(P)–Met–Gly–Trp–Met–Asp–Phe–NH_2	n.t.	3.5	< 1

Table 6—continued

	Peptide	Test preparation: Denervated gastric pouch of the dog	Dog pancreas	Guinea-pig gall bladder
18	BOC–Tyr(S)–Met–Gly–Trp–Met–Asp–Phe–NH_2	50–75	55–75	50–75
19	BOC–Tyr(S)–Thr–Gly–Trp–Met–Asp–Phe–NH_2	40–50	30–40	60–70
20	BOC–Tyr(S)–Nle–Gly–Trp–Met–Asp–Phe–NH_2	30–60	60–70	110–150
21	BOC–Tyr(S)–Tyr–Gly–Trp–Met–Asp–Phe–NH_2	20	15–50	10–20
22	BOC–Tyr(S)–Trp–Gly–Trp–Met–Asp–Phe–NH_2	10–15	10	20–35
23	BOC–Tyr(S)–Phe–Gly–Trp–Met–Asp–Phe–NH_2	10–20	10–15	10–20
24	BOC–Tyr(S)–Tyr(S)–Gly–Trp–Met–Asp–Phe–NH_2	n.t.	1.4	1
25	BOC–Tyr(S)–Thr–Gly–Trp–Nle–Asp–Phe–NH_2	120–140	130–140	130–140
26	BOC–Tyr(S)–Abu–Gly–Trp–Nle–Asp–Phe–NH_2	n.t.	15–20	40–60
27	Hcy(S)–Abu–Gly–Trp–Nle–Asp–Phe–NH_2	n.t.	3–5	2–6
28	Hpp(S)–Abu–Gly–Trp–Nle–Asp–Phe–NH_2	30	30	30–60
29	BOC–(D)Tyr(S)–Val–Gly–Trp–Met–Asp–Phe–NH_2	20–22	6–15	5–7
30	BOC–*m*.Tyr(S)–Val–Gly–Trp–Met–Asp–Phe–NH_2	2	2–2.5	<1
31	BOC–3Cl.Tyr(S)–Val–Gly–Trp–Met–Asp–Phe–NH_2	7–8	20–25	10–15
32	BOC–3,5Br.Tyr(S)–Val–Gly–Trp–Met–Asp–Phe–NH_2	2	7–8	<1–1.5
33	Phe(S)–Thr–Gly–Trp–Met–Asp–Phe–NH_2	10–20	10–20	10
34	Phe(SO_2NH_2)–Thr–Gly–Trp–Met–Asp–Phe–NH_2	n.t.	0.5–1	<1

BOC,*tert*-butyloxycarbonyl; Pyr,pyroglutamic acid; Tyr(S), tyrosine *O*-sulphate; Tyr(P), tyrosine *O*-phosphate;*m*.Tyr,*m*.tyrosine; 3Cl.Tyr,3-chloro-tyrosine; 3,5 Br.Tyr, 3,5-dibromo-tyrosine; Hcy, *p*-hydroxycinnamic acid; Hpp, *p*-hydroxyphenylpropionic acid; Phe(S), *p*-sulphonylphenylalanine; Phe(SO_2NH_2), *p*-sulphonamidophenylalanine; n.t., not tested.

phosphoric acid yielded considerably less active compounds. Similarly, substitution of *p*-sulphonyl-phenylalanine for tyrosine *O*-sulphate caused a 50 to 80 per cent reduction of activity. Desamination of tyrosine *O*-sulphate to *O*-sulphate of *p*-hydroxyphenylpropionic acid, on the contrary, produced no change in biological activity. However, the *p*-tyrosyl sulphate residue could not be replaced by the *m*-tyrosyl sulphate residue. Finally, substitution of L-tyrosine *O*-sulphate with D-tyrosine *O*-sulphate produced a reduction of activity of different intensity, depending on the test preparation.

(iii) Substitution of the threonyl residue in the C-terminal heptapeptide produced different results, depending on the entering amino acid. Tryptophan, phenylalanine and still more glycine and tyrosine *O*-sulphate gave unfavourable results, whereas methionine produced an increase in biological activity, and tyrosine caused only a minor decrease in activity. It is evident from these results that the negative charge at the N-terminus must not exceed certain limits.

(iv) Omission of the threonyl residue, with consequent shifting of the sulphated tyrosyl residue to position 6, as in the C-terminal hexapeptide of gastrin II, produced a peptide which still retained a considerable activity on gastric and pancreatic secretions, but was deprived of any significant action on the smooth muscle of the intestines and gall bladder as well as on systemic blood pressure.

(v) Omission of the glycyl residue, again with shifting of the sulphated tyrosyl residue to position 6, produced a hexapeptide possessing a moderate activity on gastric secretion (5 to 10 per cent), a poor activity on pancreatic secretion and virtually no activity on vascular and extravascular smooth muscle.

(vi) The tryptophanyl residue could not be replaced by a phenylalanyl residue.

(vii) The easily oxidized methionyl residue could be replaced, with advantage, by the stable norleucyl residue, as in the case of gastrin analogues[106].

(viii) The C-terminal tetrapeptide and the C-terminal pentapeptide of caerulein, which incidentally are also the C-terminal tetrapeptide and pentapeptide of the gastrins I and II, were completely devoid of any cholecystokinetic activity and of any stimulant activity on the intestinal smooth muscle, but still retained some secretagogue activity on the pancreas (< 3 per cent) and the rat stomach (1 to 2 per cent). The same was true for the C-terminal hexapeptide of caerulein.

The above conclusions have been largely confirmed using other test systems. Even in the isolated mucosa of the frog stomach, using the 'short circuit current' method, a prerequisite for activity of caerulein-like peptides was the occurrence of a sulphated tyrosyl residue at position 7. The hexapeptide of gastrin II possessed barely 2 to 3 per cent of the activity of caerulein, and gastrin I, like all other desulphated peptides of this group, less than 0.2 per cent of the caerulein activity[90].

These results point to the possibility that gastrins in lower vertebrates may be related in their amino acid composition and sequence (especially in the position of their probable sulphated tyrosyl residue) more closely to the caeruleins than to the gastrins.

It has been demonstrated, quite recently, that even the C-terminal dipeptide

of caerulein, Asp–Phe–NH_2, displayed some pharmacological actions on certain preparations of intestinal smooth muscle. On the isolated guinea-pig ileum, for example, the dipeptide showed 0.2 to 0.3 per cent of the activity of caerulein, on a molar basis, and as much as 2 per cent of the activity of caerulein on the pylorus of the rat stomach *in situ*. This is another striking example of the pharmacological possibilities of the oligopeptides, in this case of the smallest possible oligopeptide.

Needless to say the results obtained in the study of caerulein-like peptides are fully valid also for cholecystokinin and, subordinately, for the gastrins. The converse is similarly true, because each peptide of one of the three series is similar to the peptides of the two other series.

BOMBESIN-LIKE PEPTIDES

Three polypeptides belonging to this family have been recently isolated in a pure form, reproduced by synthesis, and submitted to a pharmacological study by two groups of research workers, independently[107–110]. From the formulae below it is evident that we have to do with a new class of polypeptides.

(I) Pyr–Gln–Arg–Leu–Gly–Asn–Gln–Trp–Ala–Val–Gly–His–Leu–Met–NH_2
(II) Pyr–Gly–Arg–Leu–Gly–Thr–Gln–Trp–Ala–Val–Gly–His–Leu–Met–NH_2
(III) Pyr————————Val–Pro–Gln–Trp–Ala–Val–Gly–His–Phe–Met–NH_2

Bombesin (I) is a tetradecapeptide obtained from methanol extracts of the skin of the two European discoglossid frogs *Bombina bombina* and *Bombina variegata variegata* where it is contained in amounts ranging from 200 to 700 $\mu g\ g^{-1}$ fresh tissue. It is highly probable that authentic bombesin is present also in the skin of *Bombina variegata pachypus*.

Alytesin (II) is again a tetradecapeptide, strictly related to bombesin, which is found in extracts of the skin of another European discoglossid frog, *Alytes obstetricans*, in amounts usually ranging between 600 and 1300 $\mu g\ g^{-1}$ wet skin, but sometimes as low as 50 $\mu g\ g^{-1}$, when expressed in terms of bombesin[108].

Discoglossus pictus, from both Sardinia and Sicily, although belonging to the same family as *Bombina* and *Alytes*, did not contain detectable amounts of bombesin-like peptides.

Finally, ranatensin (III) is an endecapeptide prepared from extracts of the skin of the American frog *Rana pipiens*. No quantitative data on ranatensin contents in the skin were presented by Nakajima *et al.*[107]. In our methanol extracts of dried skins of *Rana pipiens*, the content of ranatensin, assayed on rat uterus preparation and expressed in terms of bombesin, ranged between 0.5 and 120 $\mu g\ g^{-1}$ dry tissue[111].

From the formulae above it may be seen that the three peptides have in common the pyroglutamyl residue at the N-terminus and the C-terminal octapeptide with the sole exception, for ranatensin, of a phenylalanyl residue replacing the leucyl residue, in position 2 from the C-terminus.

Bombesin and alytesin could be easily demonstrated on paper chromatograms and electropherograms of crude or, much better, semi-purified

extracts by means of colour reactions: the Pauly reaction (histidine), the coupling reaction with the NNCD reagent or *p*-dimethylaminobenzaldehyde reaction (tryptophan) and the Sachaguki reaction (arginine). Bombesin and alytesin were accompanied by other compounds giving similar colour reactions.

The first stage in the purification of bombesin-like peptides was chromatography on an alkaline alumina column, followed by elution with descending concentrations of ethanol. Bombesin and alytesin emerged in 85 to 80 per cent ethanol eluates, other bombesin-like peptides in 95 to 90 per cent ethanol eluates.

In fact, bombesin, alytesin and ranatensin are by no means the only representatives of this peptide family and the occurrence of bombesin-like peptides is not confined to discoglossid frogs and to *Rana pipiens*. At least three other bombesin-like peptides have been traced, in our screening, in amphibians of Australia and New Guinea, demonstrating that peptides of the bombesin group also have a fairly broad distribution. The elucidation of the structure of the new bombesins is in progress[112].

The spectrum of biological activity of alytesin and bombesin is characteristic, and the distinction of these polypeptides from the other polypeptides of the amphibian skin is easily accomplished by means of parallel bioassay. All the bombesin-like peptides so far tested have been found to display similar pharmacological actions, with only quantitative differences; thus, bombesin will be used as a prototype of this polypeptide group. The pharmacological study of bombesin is still in progress in several directions and here only the effects which may be considered as well established will be reported in some detail.

(i) Bombesin displayed a stimulant action on numerous preparations of intestinal, uterine and urinary tract smooth muscle. Sometimes the effect was easily repeatable and showed a fair proportionality to the dose, but at other times a prompt and intense tachyphylaxis was observed[111, 113, 114].

On the oestrous uterus of the rat, bombesin was approximately as active as bradykinin and oxytocin, and 3 to 5 times more potent than Val^5–angiotensin II. The threshold dose was of the order of 0.005 to 0.05 ng ml^{-1} nutrient liquid and there was a good dose–response relationship. With large doses of bombesin the increase in tone often persisted for hours, in spite of repeated washing of the organ with fresh nutrient liquid.

Although the *in vivo* pregnant uterus contracted in response to ranatensin or bombesin, the foetuses did not abort. In fact, the normal gestation periods of rats in the 18th, 20th and 21st day of pregnancy was not altered either by intravenous infusion of 1 μg kg^{-1} min^{-1} of ranatensin for 2 to 3 hours or by single intravenous doses of 10 μg administered every 10 minutes for the same period[114].

On the kitten small-intestine bombesin was again approximately as active as bradykinin (threshold 0.1 to 0.5 ng ml^{-1}), but far more potent than most biogenic substances known to stimulate smooth muscle, including Val^5–angiotensin II. For this reason and because of the frequent lack of tachyphylaxis the kitten small intestine must be included among the most suitable preparations for the quantitative assay of bombesin-like peptides.

Two additional preparations can be recommended: the guinea-pig colon

(threshold 0.03 to 0.5 ng ml^{-1}) and the rat urinary bladder, both isolated (threshold 0.2 to 1 ng ml^{-1}) and *in situ*. The last preparation responded to bombesin with a long-lasting increase in tone, often accompanied by reinforcement of movements. The threshold dose for rapid intravenous injection was 50 to 100 ng kg^{-1}, for intravenous infusion 30 to 50 ng kg^{-1} min^{-1}, and for subcutaneous injection 3 to 10 μg kg^{-1}. With 100 μg kg^{-1} a spasm of the urinary bladder could be observed, lasting longer than 3 to 4 hours.

Other smooth-muscle preparations were sensitive to bombesin but owing to prompt and intense tachyphylaxis they were unsuitable for the bioassay of the polypeptide (guinea-pig ileum, rat large intestine, rabbit duodenum and colon, guinea-pig urinary bladder and ureter); still other preparations were poorly sensitive or insensitive (rabbit, cat, guinea-pig and hamster uteri; hamster, chicken and frog intestinal loops, rat ureter, dog urinary bladder and ureter, monkey urinary bladder). Like bradykinin, bombesin caused a prevalent relaxation of the rat duodenum.

Bombesin seems to act on extravascular smooth muscle mainly by a direct effect. However, in some cases cholinergic and adrenergic mechanisms cannot be excluded.

(ii) In most species (dog, cat, rabbit, rat and chicken) bombesin elicited moderate hypertension, rarely exceeding 40 to 50 mmHg, which was usually gradual in onset and slow to disappear. Tachyphylaxis was frequent. During an intravenous infusion of bombesin in the dog (threshold 1 to 3 ng kg^{-1} min^{-1}) the rise in blood pressure could sometimes be maintained at a steady level as long as the infusion was continued, but at other times the rise of pressure slowly subsided with continued administration of the polypeptide. In the rat and the chicken hypertension elicited by high doses of bombesin was often followed by secondary hypotension. Bombesin-induced hypertension was apparently not affected by pretreatment with either α- or β-adrenergic blocking agents. Similarly, secondary hypotension was not abolished by atropine. Thus, the effect of bombesin on vascular smooth muscle seems to be predominantly a direct one. Angiotensin was usually more potent than bombesin, and its effect on blood pressure was more rapid and of shorter duration.

In sharp contrast to other species, the monkey responded to bombesin with frank hypotension (threshold 2 to 10 ng kg^{-1}, by rapid intravenous injection), which was usually proportional to the dose, and which was equal to, or greater than, that caused by eledoisin or physalaemin, and of longer duration. Tachyphylaxis was moderate for low and adequately spaced doses of the polypeptide, but prompt and intense for high doses. Long-lasting hypotension was obtained by intravenous infusion of bombesin (threshold 1 to 2 ng kg^{-1} min^{-1}), but repeated infusions caused tachyphylaxis[115].

(iii) Bombesin caused in the anaesthetized dog a potent antidiuretic effect, up to complete arrest of urine flow. Threshold doses, by intravenous infusion, were of the order of 0.5 to 1 ng kg^{-1} min^{-1}. Antidiuresis was the result of a reduction in glomerular filtration rate caused by a fall in intraglomerular hydrostatic pressure. This, in its turn, was provoked by afferent vasoconstriction. The spasmogenic effect of bombesin on the smooth muscle of the afferent arterioles was directly demonstrated by the radioactive microspheres technique and indirectly by the ^{85}Kr washout method and the ^{3}H-*p*-amino-

hippurate clearance. The vascular compartment most sensitive to bombesin was that of the outer cortical zone, especially in its external half. Filtration fraction decreased under the influence of bombesin, indicating that the effect of the polypeptide on postglomerular arterioles was, if present, only of minor importance.

At an infusion rate of 12 ng kg^{-1} min^{-1} glomerular filtration rate was virtually abolished whereas blood flow in the outer cortex and in the juxtamedullary cortex, as measured by the radioactive microspheres method, was reduced by 70 and 57 per cent, respectively.

Radioautographs of the kidney after injection of ^{85}Kr confirmed that washout of the gas was strikingly retarded following infusion of bombesin. For example, a similar pattern of distribution of radioactivity in external cortex was seen after 15 seconds in the kidney of control dogs, and after 4 minutes in the kidney of bombesin-treated animals.

At high infusion rates (above 6 ng kg^{-1} min^{-1}), bombesin produced a decrease in ^{3}H-*p*-aminohippurate extraction. The effect of the polypeptide on fractional distal delivery of sodium varied depending on the dose: at low infusion rates it decreased, at high infusion rates it increased. The total glucose appearing in urine following a glucose load was sharply reduced by bombesin. However, the ratio GRF:TmG, i.e. the concentration of glucose in urine, did not show any appreciable change. It is highly probable that the above events largely depend on the vascular effects of the polypeptide, but the possibility that they are in part due to a direct action of bombesin on the tubules cannot be excluded. Research is in progress to elucidate this crucial point[116, 117].

(iv) Afferent vasoconstriction produced by bombesin was accompanied by a conspicuous activation of the renin–angiotensin system and, if sufficiently prolonged and kept within certain limits, by a considerable release of erythropoietin[116–118].

Activation of the renin–angiotensin system was observed at threshold infusion rates less than 3 ng kg^{-1} min^{-1}. There was an increase in renin secretion, followed by increases of renin activity and angiotensin II concentrations in systemic arterial blood. At an infusion rate of 6 ng kg^{-1} min^{-1} renin secretion rose by 3 to 20 times, renin activity in arterial blood by 2 to 4 times, and finally angiotensin II concentration in arterial blood by 2 to 6 times. When bombesin was infused into one renal artery only the infused kidney presented afferent vasoconstriction and increased renin secretion.

The pattern of renin secretion under the influence of bombesin was different depending on the infusion rates of the polypeptide. At low infusion rates an increased renin secretion was seen throughout the infusion period, at high infusion rates two peaks of renin secretion could be observed, one at the beginning of the infusion, the other soon after the infusion had been discontinued. Arrest or reduction of renin discharge from the juxtaglomerular apparatus during bombesin infusion may be interpreted as a direct consequence of afferent vasoconstriction which slows down, owing to lack of *vis a tergo*, the flow of fluid through the interstitial space of the juxtaglomerular apparatus into which renin is released from the granular cells.

As already stated, a prolonged infusion of bombesin caused in the dog the liberation of erythropoietin from renal tissue[118]. 3 ng kg^{-1} min^{-1} infused

over a 6-hour period was virtually ineffective; 6 ng kg^{-1} min^{-1} produced a maximum erythropoietin release, up to 4- to 6-times the basal values; with 12 ng kg^{-1} min^{-1} erythropoietin release was considerably less pronounced. Erythropoietin in the dog blood began to increase 2 hours after starting the bombesin infusion, but increase outlasted interruption of infusion by more than 6 hours.

The causal agent for erythropoietin release is considered to be hypoxia. When this was mild (low rates of bombesin infusion) no evident erythropoietin release occurred; when it was exceedingly severe (excessive afferent vasoconstriction following high rates of bombesin infusion) erythropoietin release decreased.

From preliminary experiments it seems that liberation of erythropoietin and liberation of renin are independent events. In fact, at the time of maximum erythropoietin concentration no excess of renin activity was found in blood. This would indicate that the site of renin production in the kidney is different from the site of erythropoietin production.

During the infusion of bombesin in the dog (6 ng kg^{-1} min^{-1} for 6 hours), oxygen consumption by the renal tissue decreased in parallel to the renal blood flow. However, oxygen extraction (i.e. arterio-venous oxygen difference) was critically dependent on the magnitude of the reduction in renal blood flow. When passage of blood through the kidney remained above 1 ml g^{-1} min^{-1} no appreciable changes in oxygen extraction occurred, in spite of a 60 to 80 per cent decrease in oxygen consumption; when renal blood flow fell below 1 ml g^{-1} min^{-1}, oxygen extraction clearly increased, to return again to basal values as soon as a renal blood flow exceeding 1 ml g^{-1} min^{-1} was restored[118].

(v) Although the actions of bombesin on the kidney are presently those most thoroughly studied, another important target organ for bombesin has emerged, quite unexpectedly, from the pharmacological screening. It is the stomach, especially that of the dog[119].

Bombesin may be considered a formidable stimulant of acid secretion in the denervated fundic pouch of the conscious dog. By subcutaneous injection the threshold dose of bombesin was of the order of 5 to 10 ng kg^{-1}, which is ten times less than the threshold dose of caerulein. A clear dose–response relationship could be seen up to an optimum of 2 μg kg^{-1}, where both volume and acid outputs attained peak values which were 6- to 20-times the basal values. For large doses of bombesin the effect lasted more than 4 to 5 hours. The concentration of hydrochloric acid in the gastric juice was 3 to 5 times above the basal values throughout the period of secretory response, whereas concentration of pepsin was always below the basal values. The threshold dose of bombesin capable of producing an appreciable increase in acid gastric secretion by intravenous infusion was 0.05 μg kg^{-1} h^{-1} and the effect was proportional to the dose up to 1 μg kg^{-1} h^{-1}.

In contrast to gastrin and caerulein, bombesin stimulated acid secretion by the dog stomach even when given by rapid intravenous injection. However, by this route of administration the polypeptide was less active than by either subcutaneous injection or intravenous infusion.

Atropine nearly completely inhibited the gastric secretory response to bombesin.

The polypeptide was a poor and irregular stimulant of the acid secretion in the perfused stomach preparation of the rat. Preliminary experiments in the chicken seem, on the contrary, to indicate that in this species bombesin is a good gastric secretagogue.

Finally, bombesin produced an increase in the 'short circuit current' of the isolated gastric mucosa of *Rana esculenta*, which was only 0.1 per cent of that produced by caerulein[120].

Research is in progress to elucidate the mechanism by which bombesin stimulates gastric acid secretion. There is strong experimental evidence that bombesin is a potent releaser of gastrin from the dog and man antral mucosa. In fact, intravenous infusions of bombesin produced in both species a conspicuous increase of immunoreactive plasma gastrin, which was particularly intense when acidification of the antrum was hindered and was lacking or strongly reduced in dogs and human patients subjected to antrectomy.

The panorama of the pharmacological actions of bombesin, although already broad, is far from being complete. Apart from the fact that the study of the effects of the polypeptide in the stomach and especially in the kidney may be considered little more than commenced, new actions of bombesin are emerging as our screening procedures are improving and expanding.

Available pharmacological data concerning ranatensin are limited to the effects of the polypeptide on some isolated smooth muscle preparations and on blood pressure of the common laboratory animals. On the whole ranatensin showed, as expected, a spectrum of biological activity very similar to that of bombesin. In fact, the peptide displayed a stimulant action on the guinea-pig ileum and the rat uterus and a relaxant effect on the rat duodenum. In addition it produced a relatively weak sustained contraction of the rabbit aortic strip, but not of the rat aortic strip[114].

Ranatensin raised blood pressure in the dog and rabbit, where it showed 10 per cent of the potency of angiotensin, but did not alter blood pressure in cats and had a variable action in the guinea-pig and rat. The peptide lowered blood pressure in the monkey, being as potent as eledoisin (threshold by intravenous injection, 2 to 9 ng kg^{-1}). In the dog the threshold intravenous doses were similar (10 ng kg^{-1}) for angiotensin and ranatensin, but the dose–response curve for ranatensin was more horizontal and the duration of action substantially longer.

Both hypertensive and hypotensive responses elicited by ranatensin are believed to be the result of a direct effect of the peptide on vascular smooth muscle[121, 122].

So far, only a relatively small number of bombesin-like peptides has been prepared by synthesis, essentially with the aim of determining the minimum length of the amino acid chain required for the first appearance of the bombesin effects on different smooth muscle preparations, and the minimum length necessary for maximal effects on the same preparations. Results are in part shown in *Table 7*. The activity of bombesin was set equal to 100, that of the bombesin-like peptides was expressed as a percentage.

From the comparative bioassay of bombesin-like peptides the following conclusions may be drawn:

(a) bombesin-like effects began to appear in the C-terminal heptapeptide.

Table 7. Relative potency of bombesin-like peptides, on a weight basis (bombesin = 100)

Peptides	Test preparations			
	Guinea-pig large intestine	Cat small intestine	Rat uterus	Rat urinary bladder
Pyr–Gln–Arg–Leu–Gly–Asn–Glu–Trp–Ala–Val–Gly–His–Leu–Met–NH_2 Bombesin	100	100	100	100
Pyr–Gly–Arg–Leu–Gly–Thr–Glu–Trp–Ala–Val–Gly–His–Leu–Met–NH_2 Alytesin	50–150	70–300	70–100	n.t.
Ala–Val–Gly–His–Leu–Met–NH_2	<0.1	<0.1	<0.1	<0.1
BOC–Ala–Val–Gly–His–Leu–Met–NH_2	<0.1	<0.1	<0.1	<0.1
Trp–Ala–Val–Gly–His–Leu–Met–NH_2	0.1–0.5	<0.1–0.3	2.5–10	0.2–0.5
BOC–Trp–Ala–Val–Gly–His–Leu–Met–NH_2	1–2	0.5–1	40–50	6–14
Glu–Trp–Ala–Val–Gly–His–Leu–Met–NH_2	2–8	1–4	20–30	2–10
BOC–Glu–Trp–Ala–Val–Gly–His–Leu–Met–NH_2	25–60	40–100	40–75	30–60
Asn–Glu–Trp–Ala–Val–Gly–His–Leu–Met–NH_2	100–120	100–150	150–300	150–300
Thr–Glu–Trp–Ala–Val–Gly–His–Leu–Met–NH_2	100–140	100–150	150–400	150–350
BOC–Asn–Glu–Trp–Ala–Val–Gly–His–Leu–Met–NH_2	110–220	110–160	160–500	300–500
Gly–Asn–Glu–Trp–Ala–Val–Gly–His–Leu–Met–NH_2	110–200	90–200	120–200	100–150
BOC–Gly–Asn–Glu–Trp–Ala–Val–Gly–His–Leu–Met–NH_2	30–60	30–110	30–60	25–40
Leu–Gly–Asn–Glu–Trp–Ala–Val–Gly–His–Leu–Met–NH_2	100–160	80–150	50–200	100–170
Leu–Gly–Thr–Glu–Trp–Ala–Val–Gly–His–Leu–Met–NH_2	80–130	80–150	45–180	60–100
BOC–Leu–Gly–Asn–Glu–Trp–Ala–Val–Gly–His–Leu–Met–NH_2	120–210	60–120	35–130	70–120

Pyr,pyroglutamic acid; BOC, ter-butyloxycarbonyl.

The hexapeptide was fully inactive (<0.1 per cent). The preparation most sensitive to the heptapeptide was the rat uterus.

(b) addition of the glutamine residue to the N-terminus of the heptapeptide produced a 5- to 10-fold increase of activity on all tested preparations. Addition of the glycine residue to the N-terminus of the octapeptide likewise produced a striking increase of activity on all tested preparations. The C-terminal nonapeptide of bombesin was as active as, or even more active than, bombesin itself.

(c) protection of the N-terminal alanine residue of the hexapeptide with a *tert*-butyloxycarbonyl group (BOC-hexapeptide) did not cause the appearance of any activity. However, protection with BOC of the N-terminal tryptophan residue of the heptapeptide (BOC–heptapeptide) produced 5- to 20-fold increases of activity, yielding a compound which was as active as the octapeptide itself; similarly the BOC–octapeptide was several times more potent than the octapeptide, approaching the activity of the nonapeptide. The BOC–nonapeptide, on the contrary, was approximately as active as the nonapeptide, and the BOC–decapeptide consistently less active then the decapeptide.

Thus, the presence of the tryptophan residue seemed necessary for the appearance of the bombesin-like activity. Tryptophan could not be substituted by the BOC group. However, further lengthening of the peptide chain from the hepta- to the nona-peptide, which caused striking increase in activity up to an optimum in the nonapeptide, could be obtained not only by addition of amino acid residues, but also by the addition of the BOC group.

(d) no appreciable differences in activity could be seen between the nona- and the deca-peptide, and between the deca- and the endeca-peptide. These peptides were also hardly distinguishable from each other from a qualitative point of view, for example in the appearance of tachyphylaxis and in the shape of the response.

(e) substitution in the C-terminal nonapeptide of bombesin of threonine for asparagine, as in alytesin, did not change biological activity.

(f) replacement in the C-terminal octapeptide of bombesin of glutamic acid by asparagine or valine produced no changes in activity, except perhaps in the stimulant action on the rat uterus, which appeared to be increased. Replacement of glutamic acid by phenylalanine, on the contrary, increased the activity on all tested preparations.

As previously stated, the isolation of new natural bombesin-like peptides is in progress. It may be anticipated that they will differ consistently from bombesin in the intensity and duration of their effects on different test preparations. It is possible that they may represent models for the synthesis of bombesin-like peptides possessing peculiar characteristics.

Bombesin and bombesin-like peptides occupy in some respects a unique position among the active peptides of the amphibian skin. In fact, whereas all other hitherto described polypeptide groups of the amphibian skin have their counterpart in peptides occurring in mammalian tissues, this does not occur for bombesin-like peptides. The bradykinins of the amphibian skin have their duplicate in the mammalian plasma kinins, the physalaemin-like peptides of the amphibian skin are strictly related to substance(s) P of the mammalian brain and gastro-intestinal tract, and finally the caeruleins bear the strictest chemical and biological resemblance to cholecystokinin and, subordinately, to the gastrins.

So far a counterpart of the bombesin-like peptides is lacking in the mammalian organism. The question is whether this lack is a real one or whether it simply depends on the fact that bombesin-like peptides have never been sought with suitable methods. Our research group is presently trying to solve this problem.

MISCELLANEOUS POLYPEPTIDES

In addition to the previously described polypeptides which must be considered as firmly established chemical and pharmacological entities, several other active peptides have been traced in the skin of different amphibian species.

For example, two peptides mimicking, on the whole, physalaemin in their pharmacological effects, but certainly different from the tachykinins, have been traced in the skin of African and Australian amphibians, respectively.

Another peptide producing prolonged hypotension and possibly acting also as a releasing factor on endocrine glands has been found in a South American frog.

A major obstacle for the isolation and study of these peptides is the scarcity of material. However, sometimes there are also serious methodological difficulties, due to the considerable length of the amino acid chain.

The polypeptides found by Michl and coworkers[124–126] in the cutaneous venom of *Bombina variegata variegata* deserve a particular mention. So far the following peptides have been isolated in a pure form:

(I) Ala–Glu–His–Phe–Ala–Asp–NH_2
(II) Ser–Ala–Lys–Gly–Leu–Ala–Glu–His–Phe
(III) Gly–Ala–Lys–Gly–Leu–Ala–Glu–His–Phe
(IV) Gly–Ile–Gly–Ala–Leu–Ser–Ala–Lys–Gly–Ala–Leu–Lys–Gly–Leu–Ala–Lys–Gly–Leu–Ala–Glu–His–Phe–Ala–Asp–NH_2 Bombinin

It may be seen that peptide (I) is nothing but the C-terminal hexapeptide of bombinin and, similarly, that peptides (II) and (III) are strictly related to each other and to bombinin. These peptides, on the other side, have nothing to do with bombesin-like peptides.

Very little is known about the pharmacological properties of bombinin, apart from the indication that it is endowed with a potent haemolytic action.

DISCUSSION

It has been repeatedly pointed out that polypeptides herein described represent only a part of the active peptides occurring in amphibian skin. Not only do several already identified peptides still await isolation and thorough pharmacological study, but it is highly probable that other peptides are escaping our attention because their activity lies beyond the limits of our screening methods, although these are covering a progressively increasing number of pharmacological effects.

As previously noted, amino acid sequences found in amphibian skin are the repetition of identical or similar sequences present in mammalian tissues. There are sound reasons to believe that elucidation of the structure of other amphibian peptides will confirm this statement.

Why does the genetic code cause the allineation in the same sequence of the same amino acid residues in amphibian skin, in the posterior salivary glands of octopods and in different, sometimes highly differentiated tissues of mammals? What is the origin, the significance and the function of active peptides in amphibian skin?

These questions must be left open until the analytic, descriptive phase of research is more advanced and until sufficient information is available about the biosynthesis and fate of the polypeptide molecules.

For example we have at present no idea whether amphibian peptides are the result of a progressive aggregation of smaller peptides, single amino acid residues or both, or of a splitting or hydrolysis of larger peptides. Both peptide fragments and larger precursors have been occasionally traced in the skin. Research on the occurrence in the cutaneous tissue of enzyme systems

catalyzing on the one side the formation of active peptides and on the other side their breakdown and inactivation is completely lacking.

Anyhow, it seems probable that peptides present in methanol extracts pre-exist in the living skin in a free form or very loosely bound. In fact, by means of an injection of adrenaline into the lymphatic dorsal sac of a *Xenopus* it was possible to produce the prompt appearance of large amounts of caerulein in the water in which the frogs were immersed. This experiment shows two things: first that caerulein is ready for secretion in the skin, and secondly that it may be secreted externally together with the other components of the so called 'cutaneous venom'. The fact that polypeptides in the skin are mainly localized in the cutaneous glands, the secretion of which is generally held to be only external, and that peptides actually appear in the cutaneous secretion, does complicate the understanding of the possible significance and function of these extremely active molecules in the amphibian skin. A true endocrine function, following their discharge into the blood stream, lacks at present any experimental evidence. The intervention of the skin polypeptides in local regulation seems more conceivable. It has been tentatively suggested that they may interfere in some basic functions of the skin, for example in the regulation of the secretion of the skin or in the control of water and electrolyte exchanges through the skin.

It is obvious that the same function may be displayed in the different amphibian species by different polypeptides, and it is possible that polypeptides inactive in our screening systems are active in amphibian skin.

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REFERENCES

[1] V. Erspamer, *Ann. Rev. Pharmacol.* **11**, 327 (1971).
[2] A. Anastasi, V. Erspamer and J. M. Cei, *Arch. Biochem. Biophys.* **108**, 341 (1964).
[3] G. Bertaccini, J. M. Cei and V. Erspamer, *Br. J. Pharmac.* **25**, 363 (1963).
[4] A. Anastasi and G. Falconieri Erspamer, *Experientia* **26**, 866 (1970).
[5] V. Erspamer, G. De Caro and R. Endean, *Experientia* **22**, 738 (1966).
[6] A. Anastasi and V. Erspamer, *Arch. Biochem. Biophys.* **101**, 56 (1963).
[7] M. M. Chang and S. E. Leeman, *J. Biol. Chem.* **245**, 4784 (1970).
[8] M. M. Chang, S. E. Leeman and H. D. Niall, *Nature (New Biology)* **232**, 86 (1971).
[9] G. Bertaccini, J. M. Cei and V. Erspamer, *Br. J. Pharmac.* **25**, 380 (1963).
[10] G. B. Fregnan and A. Glaesser, *Arch. Int. Pharmacodyn.* **171**, 435 (1968).
[11] M. Bergamaschi and A. H. Glaesser, *Circulation Res.* **15**, 391 (1964).
[12] W. Lochner and J. R. Parratt, *Br. J. Pharmac.* **26**, 17 (1966).
[13] J. Nakano, B. A. Darrow and J. R. McCurdy, *Arch. Int. Pharmacodyn.* **172**, 429 (1968).
[14] H. A. Kontos, W. Shapiro, H. P. Mauck and J. L. Patterson jr., *J. Appl. Physiol.* **19**, 113 (1964).
[15] G. Bertaccini and G. De Caro, *J. Physiol. (London)* **181**, 68 (1965).
[16] F. Lembeck, F. Geipert and K. Starke, *Naunyn-Schmiedebergs Arch. Pharmak. exp. Path.* **261**, 422 (1968).
[17] G. De Caro and M. Cordella, *Ann. Oftalmol. Clin. ocul.* **91**, 933 (1965).

[18] N. Emmelin, P. Ohlin and A. Thulin, *Br. J. Pharmac.* **37**, 666 (1969).
[19] Ch. A. Schneyer and H. D. Hall, *Proc. Soc. Exp. Biol. Med.* **127**, 1245 (1968).
[20] G. Bertaccini, G. De Caro and M. Impicciatore, *J. Physiol. (London)* **193**, 487 (1967).
[21] G. Zetler, D. Mönkemeier and H. Wiechell, *Naunyn-Schmiedebergs Arch. Pharmak. exp. Path.* **262**, 97 (1969).
[22] G. Falconieri Erspamer, L. Negri and D. Piccinelli, *Naunyn-Schmiedeberg's Arch. Pharmak. exp.* in press.
[23] P. Mantovani, G. L. Piccinin and G. Bertaccini, *Pharmacol. Res. Commun.* **1**, 172 (1969).
[24] G. De Caro, *Arch. Int. Pharmacodyn.* **146**, 27 (1963).
[25] E. Stürmer and B. Berde, *J. Pharmacol. Exp. Ther.* **140**, 349 (1963).
[26] T. G. Kantor, M. E. Jarvik and B. B. Wolff, *Proc. Soc. Exp. Biol. Med.* **126**, 505 (1967).
[27] S. H. Ferreira and J. R. Vane, *Br. J. Pharmac.* **30**, 417 (1967).
[28] H. Broghammer, *Klin. Wschr.* **41**, 1097 (1963).
[29] E. F. Gersmeyer, A. Castenholz and M. Nicolay, *Klin. Wschr.* **43**, 309 (1965).
[30] F. Pratesi, A. Nuti, S. Brunetti, R. P. Dabizzi and L. Caramelli, *Folia angiologica* **3**, 266 (1966).
[31] I. Szám, D. Kusztos and G. Csapó, *Arzneimittelforschg.* **16**, 1671 (1966).
[32] G. De Caro, M. Cordella and P. Miani, *Ophthalmologica* **158**, 284 (1969).
[33] G. Falconieri Erspamer, A. Anastasi and J. M. Cei, *J. Pharm. Pharmac.* **22**, 466 (1970).
[34] B. Camerino, G. De Caro, R. A. Boissonnas, Ed. Sandrin and E. Stürmer, *Experientia* **19**, 339 (1963).
[35] E. Stürmer, Ed. Sandrin and R. A. Boissonnas, *Experientia* **20**, 303 (1964).
[36] L. Bernardi, G. Bosisio, F. Chillemi, G. De Caro, R. De Castiglione, V. Erspamer, A. Glaesser and O. Goffredo, *Experientia*, **20**, 306 (1964).
[37] L. Bernardi, G. Bosisio, F. Chillemi, G. De Caro, R. De Castiglione, V. Erspamer, A. Glaesser and O. Goffredo, *Experientia*, **21**, 695 (1965).
[38] L. Bernardi, G. Bosisio, F. Chillemi, G. De Caro, R. De Castiglione, V. Erspamer and O. Goffredo, *Experientia* **22**, 29 (1966).
[39] E. Schröder and K. Lübke, *Experientia* **20**, 19 (1964).
[40] K. Lübke, R. Hempel and E. Schröder, *Experientia* **21**, 84 (1965).
[41] E. Schröder, K. Lübke and R. Hempel, *Experientia* **21**, 70 (1965).
[42] F. Lembeck, A. Oberdorf, K. Starke and R. Hettich, *Naunyn-Schmiedebergs Arch. Pharmak. exp. Path.* **261**, 338 (1968).
[43] R. S. Dunn and A. M. Perks, *Experientia* **26**, 1220 (1970).
[44] J. J. Pisano, *Fed. Proc.* **27**, 58 (1968).
[45] A. Anastasi, V. Erspamer and G. Bertaccini, *Comp. Biochem. Physiol.* **14**, 43 (1964).
[46] A. Anastasi, V. Erspamer, G. Bertaccini and J. M. Cei, in *Hypotensive Peptides*, p. 76. (Ed. E. G. Erdös, N. Back and F. Sicuteri). Springer Verlag, New York (1966).
[47] A. Anastasi, V. Erspamer and G. Bertaccini, *Brit. J. Pharmacol.* **27**, 479 (1966).
[48] T. Nakajima, *Chem. Pharm. Bull.* **16**, 769 (1968).
[49] M. L. Roseghini and J. M. Cei, *J. Gen. Comp. Pharmacol.* **3**, 195 (1972).
[50] M. R. F. Furtado, *Biochem. Pharmacol.* **21**, 118 (1972).
[51] G. Fischer and W. Albert, *Z. Naturforschg.* **26b**, 1021 (1971).
[52] A. Anastasi, V. Erspamer and R. Endean, *Arch. Biochem. Biophys.* **125**, 57 (1968).
[53] G. De Caro, R. Endean, V. Erspamer and M. Roseghini, *Br. J. Pharmac.* **33**, 48 (1968).
[54] A. Anastasi, G. Bertaccini, J. M. Cei, G. De Caro, V. Erspamer, M. Impicciatore and M. Roseghini, *Br. J. Pharmac.* **38**, 221 (1970).
[55] A. Anastasi, G. Bertaccini, J. M. Cei, G. De Caro, V. Erspamer and M. Impicciatore, *Brit. J. Pharmacol.* **37**, 198 (1969).
[56] B. Endean, V. Erspamer, G. Falconieri Erspamer and L. Negri, To be published.
[57] V. Erspamer, G. Falconieri Erspamer, L. Negri and J. Visser, To be published.
[58] A. C. Alcala, V. Erspamer, G. Falconieri Erspamer and L. Negri, To be published.
[59] G. Bertaccini, G. De Caro, R. Endean, V. Erspamer and M. Impicciatore, *Brit. J. Pharmacol.* **34**, 291 (1968).
[60] G. Bertaccini, G. Ballarini, A. Agosti and G. Zannetti, *Arch. Int. Pharmacodyn.* **183**, 261 (1970).
[61] M. Vagne and M. I. Grossman, *Amer. J. Physiol.* **215**, 881 (1968).
[62] G. Bertaccini, T. Braibanti and F. Uva, *Gastroenterology* **56**, 862 (1969).
[63] I. Orlandini and A. Agosti, *Radiol. Medica, Torino* **55**, 1061 (1969).

[64] R. Carratù, G. Arcangeli and F. Pallone, *Rendic. Rom. Gastroenterol.* **3**, 28 (1971).
[65] A. Agosti, P. Mantovani and L. Mori, *Naunyn-Schiedebergs Arch. Pharmakol.* **268**, 114 (1971).
[66] T. M. Lin and G. F. Spray, *Gastroenterology* **56**, 1178 (1969).
[67] G. Bertaccini and A. Agosti, *Gastroenterology* **60**, 55 (1971).
[68] M. L. Ramorino, M. V. Ammaturo and F. Anzini, *Rend. Rom. Gastroenterol.* **2**, 172 (1970).
[69] M. Del Tacca, G. Soldani and A. Crema, *Agents and Actions* **1**, 176 (1970).
[70] M. Del Tacca, S. Pacini, G. Amato, C. Falaschi and A. Crema, *Europ. J. Pharmacol.* **17**, 171 (1972).
[71] A. Vizi, G. Bertaccini, M. Impicciatore and J. Knoll, *Gastroenterology* **64**, 268 (1973).
[72] A. Agosti, G. Bertaccini, R. Paulucci and E. Zanella, *Lancet* **1**, 395 (1971).
[73] G. Bertaccini, G. De Caro, R. Endean, V. Erspamer and M. Impicciatore, *Brit. J. Pharmacol.* **37**, 185 (1969).
[74] M. I. Grossman, in *Proc. Symp. Exocrine Pancreas.* Queen's University, Kingston, Ontario, Canada, June 5–7, 1969.
[75] G. F. Stening and I. M. Grossman, *Amer. J. Physiol.* **217**, 262 (1969).
[76] G. De Caro, I. Ronconi and N. Sopranzi, in *Prostaglandins, Peptides and Amines* (Symposium Florence 1968), p. 167. (Ed. P. Mantegazza and E. W. Horton), Academic Press, New York and London (1969).
[77] J. Meldolesi, *Brit. J. Pharmacol.* **40**, 731 (1970).
[78] L. Angelucci and G. Linari, *Eur. J. Pharmacol.* **11**, 204 (1970).
[79] G. Bertaccini, G. De Caro and P. Melchiorri, *Brit. J. Pharmacol.* **40**, 78 (1970).
[80] G. De Caro, G. Improta and P. Melchiorri, *Experientia* **26**, 1145 (1970).
[81] F. Fallucca, R. Carratù, G. Tamburrano, M. Javicoli, G. Menzinger and D. Andreani, *Horm. Metab. Res.* **4**, 55 (1972).
[82] L. Dorigotti and A. H. Glaesser, *Experientia* **24**, 806 (1968).
[83] G. F. Stening and I. M. Grossman, *Gastroenterology* **56**, 1047 (1969).
[84] G. Bertaccini, R. Endean, V. Erspamer and M. Impicciatore, *Brit. J. Pharmacol.* **34**, 311 (1968).
[85] L. R. Johnson, G. F. Stening and M. I. Grossman, *Gastroenterology* **56**, 1255 (1969).
[86] A. Agosti, S. Biasioli and G. Naranjo, *Boll. Soc. Ital. Biol. Sper.* **45**, 778 (1969).
[87] A. Agosti, S. Biasioli and G. Bertaccini, *Gastroenterology* **59**, 727 (1970).
[88] G. F. Stening, L. R. Johnson and M. I. Grossman, *Gastroenterology* **57**, 44 (1969).
[89] A. M. Brooks, A. Agosti, G. Bertaccini and I. M. Grossman, *New England J. Med.* **282**, 535 (1970).
[90] L. Negri and V. Erspamer, *Naunyn-Schmiedeberg's Arch. Pharmacol.* **277**, 401 (1973).
[91] P. Melchiorri and N. Sopranzi, *Pharmacol. Res. Commun.* **2**, 135 (1970).
[92] P. Melchiorri and N. Sopranzi, *Agents and Actions* **2**, 58 (1971).
[93] L. Angelucci, M. Baldieri and G. Linari, *Eur. J. Pharmacol.* **11**, 217 (1970).
[94] R. S. Jones and M. I. Grossman, *Amer. J. Physiol.* **219**, 1013 (1970).
[95] L. Angelucci, L. Micossi, F. Cantalamessa and G. Linari, *Arch. Int. Pharmacodyn.* **196** Suppl., 92 (1972).
[96] L. Angelucci, G. Linari, L. Micossi and F. Cantalamessa, *Abstr. 5th Internat. Congress Pharmacology*, p. 132. SanFrancisco, 23–28 July 1972.
[97] L. Angelucci, G. Linari, L. Micossi and F. Cantalamessa, to be published.
[98] L. Angelucci, L. Micossi and F. Cantalamessa, *Arch. Int. Pharmacodyn.* **196**, Suppl., 89 (1972).
[99] L. Angelucci, L. Micossi and M. Parri, *Abstr. 5th Internat. Congress Pharmacology*, San Francisco p. 133, 23–28 July 1972.
[100] G. Bertaccini, G. De Caro, R. Endean, V. Erspamer and M. Impicciatore, *Brit. J. Pharmacol.* **33**, 59 (1968).
[101] A. D. Care, J. B. Bruce, J. Boelkins, A. D. Kenny, H. Conaway and G. S. Anast, *Endocrinology* **89**, 262 (1971).
[102] E. Solcia, C. Capella, T. Chieli, O. Bellini and C. Bertazzoli, in *Gastrointestinal Hormones* p. 103. (Ed. L. Demling). G. Thieme Verlag, Stuttgart (1972).
[103] T. Chieli, C. Bertazzoli, G. Ferni, I. Dell'Oro, C. Capella and E. Solcia, *Toxicol. Appl. Pharmacol.* **23**, 480 (1972).
[104] A. Anastasi, L. Bernardi, G. Bertaccini, G. Bosisio, R. De Castiglione, V. Erspamer, O. Goffredo and M. Impicciatore, *Experientia* **24**, 771 (1968).

[105] L. Bernardi, G. Bertaccini, G. Bosisio, R. Bucci, R. De Castiglione, V. Erspamer, O. Goffredo and M. Impicciatore, *Experientia* **28**, 7 (1972).
[106] J. S. Morley, *Proc. 8th Europ. Peptide Symposium*, p. 226. Noordwijk-on-Sea, The Netherlands, Sept. 1966. North Holland Publishing Co., Amsterdam (1967).
[107] T. Nakajima, T. Tanimura and J. J. Pisano, *Feder. Proc.* **29**, 284 Abstr. (1970).
[108] A. Anastasi, V. Erspamer and M. Bucci, *Arch. Biochem. Biophys.* **148**, 433 (1972).
[109] A. Anastasi, *Naunyn-Schmiedebergs Arch. Pharmakol*, **269**, 135 (1971).
[110] L. Bernardi, R. De Castiglione, O. Goffredo and F. Angelucci, *Experientia*, **27**, 873 (1971).
[111] V. Erspamer, G. Falconieri Erspamer, M. Inselvini and L. Negri, *Brit. J. Pharmacol.* **45**, 333 (1972).
[112] A. Anastasi, R. Endean, V. Erspamer, G. Falconieri Erspamer and L. Negri, to be published.
[113] V. Erspamer, G. Falconieri Erspamer and M. Inselvini, *J. Pharm. Pharmacol.* **22**, 875 (1970).
[114] R. G. Geller, W. C. Govier, J. J. Pisano, T. Tanimura and V. Van Clineschmidt, *Brit. J. Pharmacol.* **40**, 605 (1970).
[115] V. Erspamer, P. Melchiorri and N. Sopranzi, *Brit. J. Pharmacol.* **45**, 442 (1972).
[116] P. Melchiorri, N. Sopranzi and V. Erspamer, *J. Pharm. Pharmacol.* **23**, 981 (1971).
[117] V. Erspamer, P. Melchiorri and N. Sopranzi, *Brit. J. Pharmacol.*, in press.
[118] P. Melchiorri and N. Sopranzi, *J. Pharmacol. Exp. Ther.* in press
[119] G. Bertaccini, V. Erspamer and M. Impicciatore, *Brit. J. Pharmacol.* in press.
[120] L. Negri, personal communication.
[121] B. V. Clineschmidt, R. G. Geller, W. C. Govier, J. J. Pisano and T. Tanimura, *Brit. J. Pharmacol.* **41**, 622 (1971).
[122] W. J. Louis, T. Tanimura and J. J. Pisano, *Eur. J. Pharmacol.* **14**, 340 (1971).
[123] R. De Castiglione, F. Angelucci, V. Erspamer, G. Falconieri Erspamer and L. Negri, to be published.
[124] G. Kiss and H. Michl, *Toxicon* **1**, 33 (1962).
[125] A. Csordás and H. Michl, *Toxicon* **7**, 103 (1969).
[126] A. Csordás and H. Michl, *Mschr. Chem.* **101**, 182 (1970).

SOME BIOLOGICAL APPLICATIONS OF REGULAR SOLUTION THEORY

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ABSTRACT

There is firm experimental and theoretical justification for expecting that solution theories, in this case regular solution theory, can be applied in a semi-empirical manner to questions of biological interest. This background is outlined and one of the possible models resulting from the use of regular solution theory is applied to the analysis of erythrocyte haemolysis. Suggestive evidence arising from correlations of *n*-octanol–water partition coefficients with parameters appropriate to this approach tends to indicate that regular solution theory may apply to a wider variety of biological systems than has previously been thought.

INTRODUCTION

Solubility-related phenomena are pervasive throughout the pharmaceutical and pharmacological literature. Among the areas where aspects of solution behaviour arise are (a) in the design of liquid dosage forms; (b) in considerations of the influence of carrier vehicles on the biological absorption or response of a drug entity; (c) in accounting for the penetrability of molecular species into or through biological tissues; and, (d) in rationalizing physicochemical influences on relative biological responses. As a more specialized component of the latter category one might also include certain factors, such as hydrophobic bonding, which relate to drug–biomacromolecule interactions. Underscoring literature accounts within each of these areas is the frequency with which simple oil–water partition coefficients are found to parallel test results obtained with biological systems. Hansch and his associates[1–3] have extensively exploited the ubiquitous occurrence of this correlation and have established statistical analyses that make use of *n*-octanol–water partition coefficients as a useful tool in the design of drug molecules or in the study of enzyme reactions. Less frequently have efforts been made to apply a theory of solution, such as regular solution theory[4, 5],

to these same types of systems. Such work as has been reported has been sporadic[6–10] although in some instances[11–19] promising results have been obtained with commerical pharmaceuticals.

There are a variety of reasons why one might prefer to make use of empirical or extrathermodynamic relations involving partition coefficients rather than relations based on a solution theory in the study of pharmaceutical or pharmacological systems: (a) empirical parameters ordinarily provide better 'fits' with biological data than do theoretically-based indexes[20]; (b) partition coefficients are approximately additive–constitutive, thus enabling the estimation of values appropriate to additional compounds[21]; (c) there are few theoretical constraints associated with the application of partition coefficients to interpretations of biological data as distribution processes involving an organic or lipophilic phase and an aqueous phase are linearly interrelated[22].

On the other hand, several valid reasons can also be given for desiring to take a more fundamentally based approach: (a) seemingly disparate experimental observations such as carrier vehicle influences on biological activity, isotonicity, and passive membrane transport are readily understood as involving a common physicochemical component; (b) the 'distribution system' is the biological assay object itself; thus, adjustable parameters within a theoretical framework have significance in relation to the actual test system; (c) based on a theoretical rationale it is sometimes possible to design independent types of assay for substantiation of a mechanistic hypothesis; (d) disagreement with theoretically-based relationships frequently are readily interpretable as providing information which is either intrinsic to the chemical nature of the compounds under consideration or is specific to biological processes; (e) in the realm of drug design, there is a potential for estimating optimum solubility characteristics without the necessity for an extensive set of test compounds and there is also afforded a possible route to the design of tissue-specific agents.

As should be evident from this brief introduction, the possible scope of application of a solution theory, in this instance regular solution theory, to biological systems is extremely broad. What we hope to do by this presentation is to report on our very preliminary findings which tend to indicate that regular solution theory might have a much more promising future in application to biological systems than has previously been thought. We do not claim our present interpretations to be immutable. Rather we anticipate that clarifications and refinements will be made in establishing a modification of regular solution theory that is appropriate to questions of biological interest.

HISTORICAL FOUNDATIONS

Virtually all efforts to relate biological activity with physically-based indexes stem from observations of a relationship between narcotic potency and water solubility[23]. Later, independent investigations led Overton[24] and Meyer[25] to the finding of a correlation between narcotic potency and partition coefficient from which it was surmised that the relative effectiveness of anaesthetic compounds was due to their distribution between biological lipids and water. This observation was extended to the penetration of

molecular species through membranes by Collander[26] and has reached fruition with the work of Hansch and his coworkers[1–3].

Meyer had a son, and it is from this Meyer's efforts that a basis for quantitative work with biological systems is derived. He showed[27] that despite the apparent trend in narcotic potency with partition coefficient, the equilibrium concentration of the drug in the organic phase of the *model* distribution system was effectively a constant. From this it was concluded that narcosis commences when any chemically indifferent substance has attained a certain molar concentration in the lipids of cells. It was also noted that this specific concentration depends on the nature of the animal or cell but is independent of the narcotic selected. More recent work by Roth and Seeman[28]

Table 1. Comparison of biological effects with partition coefficients and equilibrium concentrations in lipid.

A. Paralysis of tadpoles by narcotic agents[27]

Substance	Partition coefficient (oleyl alcohol–water)	Narcotic concentration (mol l^{-1} of water)	Oleyl alcohol conc. (mol l^{-1})
Ethanol	0.10	0.33	0.033
1-Propanol	0.35	0.11	0.038
1-Butanol	0.65	0.03	0.020
Valeramide	0.30	0.07	0.021
Antipyrine	0.30	0.07	0.021
Aminopyrine	1.30	0.03	0.039
Barbital	1.38	0.03	0.041
Benzamide	2.5	0.013	0.033
Salicylamide	5.9	0.0033	0.021
Phenobarbital	5.9	0.008	0.048
Thymol	950	0.000047	0.045

B. Antihaemolytic effect of narcotics with erythrocyte membrane[28,29]

Substance	Partition coefficient (membrane–0.4% NaCl)	Equiprotective conc. (mol l^{-1} of water)	Membrane conc. (mol kg^{-1} membrane)
Benzyl alcohol	4.0	0.022	0.089
Butanol	1.5	0.042	0.062
Pentanol	3.6	0.0145	0.053
Hexanol	13.0	0.00175	0.023
Heptanol	39.6	0.000618	0.025
Octanol	151.8	0.000235	0.036
Nonanol	582	0.0000410	0.024
Decanol	1226	0.00001	0.012
Chlorpromazine	1600	0.0000081	0.013
Morphine	7.1	0.0052	0.037
Phenol	8.5	0.0078	0.066
4-MeO-phenol	5.4	0.0060	0.033
4-F-phenol	13.1	0.0030	0.039
3-Me_2N-phenol	8.0	0.0028	0.022
4-CO_2Me-phenol	18.1	0.0024	0.044
3-NO_2-phenol	20	0·0022	0.045
4-Me-phenol	17.7	0·0014	0.025
Chloroform	18.0	0.0105	0.189
Ether	1.3	0.12	0.156
Ethanol	0.14	0.85	0.119

on the distribution of phenol anaesthetics with erythrocyte membrane has verified this conclusion, but acidic compounds that are ionized and have a negative charge under the conditions of the assay are tentatively exceptions[29]. *Table 1* compares the results of Meyer with those of Roth and Seeman to illustrate the basis for Meyer's hypothesis.

A physical rationale for Meyer's hypothesis based on thermodynamic principles was subsequently put forward by Ferguson[30] and it is this rationale which provides a foundation for applying solution theories to the interpretation of biological data. Ferguson points out that under equilibrium conditions the chemical potential for a compound in the assay medium, μ_a, should equal the chemical potential of the compound in the biophase, μ_b, irrespective of the nature of the biophase. Necessarily, then,

$$\mu_a = \mu_b^0 + RT \ln a_b \tag{1}$$

where μ_b^0 is a reference chemical potential for the drug in some standard state, and a_b is the thermodynamic activity of the drug under the experimental conditions. For perfect solutions $a_b \approx C$, the concentration measure, and hence the concentration of a substance accumulated in a common biophase is expected to be a constant and this value should be independent of the nature of the drug. If this rationale is correct, then the converse of equation 1 should also hold,

$$\mu_b = \mu_a^0 + RT \ln a_a \tag{2}$$

That is, drug activities assayed using a specific biological test system and measured to an equivalent endpoint in terms of molar concentrations or partial pressures should provide a constant value when these concentration measures are converted to thermodynamic activities. This has been found to be the case for a variety of biological assays where the test compounds are either in solution[30–34] or in a gaseous state[30, 35, 36]. *Table 2* presents an

Table 2. Toxic partial pressures and thermodynamic activities of some gaseous substances[36]

Substance	B.P., °C	Toxic partial pressure, p_t	LD_{50} (g mol × 10^{-6} l^{-1})	Thermodynamic activity, a_a
Nitrogen	−195.8	340 atm	12000000	0.28
Oxygen	−183.0	13 atm	530000	0.02
Methane	−161.5	180 atm	8800000	0.53
Argon	−185.7	92 atm	3900000	0.10
Ethylene	−103.9	15.5 atm	700000	0.31
Nitrous oxide	−89.5	14.5 atm	640000	0.36
Ethane	−88.3	11.5 atm	520000	0.37
Ethyl chloride	12.2	330 mmHg	17000	0.29
Chloroform	61.3	40 mmHg	2100	0.20
Ethylene dichloride	83.6	10 mmHg	1000	0.24
sym-Tetrachloroethane	146.3	1.7 mmHg	90	0.24

example of this type of relationship. In this case a modification of Raoult's Law is used to estimate the thermodynamic activities

$$p_t \approx p^0 a_a \tag{3}$$

where p_t is the toxic partial pressure and p^0 is the vapour pressure of the pure liquid. With solutions the thermodynamic activities are estimated[30] as the ratio S_t/S_0 of the molar concentration of the test solution S_t to the solubility S_0 of the drug.

MODELS FOR THE ANALYSIS OF BIOLOGICAL ACTIVITIES

From Ferguson's rationale it is evident that there are two alternative but equivalent expressions that can serve as a basis for the analysis of drug response A. These are (a) the distribution model suggested by Overton and Meyer

$$A = \ln (a_b/a_a) = -(\mu_b^0 - \mu_a^0)/RT \tag{4}$$

and (b) the phase saturation model advanced by Meyer's son

$$A = \mu^0 + RT \ln a \tag{5}$$

The subscript is omitted from equation 5 as this relation may apply to either phase.

Under the assumption that solubility-related phenomena account primarily for variations in many types of drug responses, equations 4 and 5 may be used to introduce parameterizations appropriate to a solution theory. This is usually done by including an appropriate expression for the thermodynamic activity coefficient γ.

Taking equation 4 as a starting point, and choosing a standard state such that $\mu_b^0 = \mu_a^0$ (e.g., pure drug), it is readily shown that

$$A = \log (S_b/S_a) = \log \gamma_a - \log \gamma_b \tag{6}$$

where S_b and S_a are molar concentrations and γ_b and γ_a are thermodynamic activity coefficients for the drug in the biophase and the assay medium, respectively. For the same choice of standard state, equation 5 would become

$$A = \log \gamma_b + \log S_b \tag{7}$$

in which S_b can be taken to be constant for a particular type of biophase.

FORMULATION IN TERMS OF REGULAR SOLUTION THEORY

A regular solution may be defined phenomenologically as having a positive heat of mixing, ΔH^M, and an entropy of mixing given by $\Delta S^M = -R \ln X_1$, where X_1 is the mole fraction of solvent. For such solutions, Hildebrand and Scott[4,5] have shown that deviations from Raoult's Law can be accounted for in many instances by expressing the thermodynamic activity coefficient as

$$\log \gamma_2 = \frac{\Phi_1^2 V_2}{2.303\, RT} (\delta_1 - \delta_2)^2 \tag{8}$$

in which V_2 is the molal volume of solute, Φ_1 is the volume fraction of solvent, and δ_1, δ_2 are the 'internal pressures' of the solvent and solute respectively. The 'internal pressure' or 'solubility parameter' δ is given strictly by the

ratio of the energy of vaporization to the molar volume of a pure substance

$$\delta = (\Delta E_v/V)^{\frac{1}{2}} = (\Delta E_v V)^{\frac{1}{2}}/V = F/V \tag{9}$$

but in many practical applications it may be estimated by making use of the approximate additive–constitutive nature of the molar attraction constant F[37,38] or by calculation from molecular polarizability[39]. Swelling, solubility and related measurements may also be made in order to estimate δ[4].

In dilute solutions $\Phi_1 \approx 1$ and if this condition applies to each of the distribution phases of a biological test system the substitution of equation 8 into equations 6 and 7 yields, respectively, the relationships

$$2.303\,RT\,A = (V_a\delta_p^2 - V_b\delta_n^2) - 2(V_a\delta_p - V_b\delta_n)\delta + (V_a - V_b)\delta^2 \tag{10}$$

$$2.303\,RT\,A = V_b\delta_n^2 - 2V_b\delta_n\delta + V_b\delta^2 + 2.303\,RT\log S_b \tag{11}$$

where δ is the solubility parameter for drug and δ_p, δ_n are *apparent* solubility parameters for the phases of the assay system. Multicomponent phases have their solubility parameter expressed in terms of the solubility parameters for the individual components by the relationship[5]

$$\delta_{app} = \sum \Phi_i\delta_i \tag{12}$$

so that the apparent solubility parameters in equations 10 and 11 may be interpreted in terms of the components making up the assay medium or the biophases. V_a and V_b are molal volumes appropriate to the drug in each phase. To an additional degree of approximation these may be considered essentially constant for a given series of drug molecules. Hence, equations 10 and 11 could be used, if desired, as model equations in a regression analysis such as is done by Hansch and his associates[1–3]. In a later section this type of approach is followed.

One advantage of having interpreted Ferguson's relationships in terms of regular solution theory is that a parabolic relationship between drug activity and drug solubility is a natural consequence of the theory. Such parabolic relationships are frequently observed and with extrathermodynamic types of approaches[1–3] had to be taken into account by virtue of a statistical expedient, i.e., the essentially arbitrary introduction of higher-powered (squared) terms into the model equation. With biological systems a variety of alternative possibilities exist that could also lead to a parabolic curve, but for the present it will be assumed that solubility alone leads to this type of curve.

A second advantage, pointed out by Mullins[6], is that optimum solubility in a particular phase is achieved when the δ values for the drug and the biophase are equal or at a minimum value. Hence, presuming δ values for certain tissues have been established empirically or by some other means one could hope to localize a drug predominantly in one tissue by attempting to match the δ value of the drug with that of the tissue. The additive–constitutive nature of molar attraction constants F and molar volumes, at least for molecules that are not strongly self-associated due to hydrogen bonding or to their charge characteristics[37–39], allow this approach to be of some value in drug design. A possible shortcoming to this concept, however, is that δ values tend to vary over a relatively narrow range (4.0 to 23) and as a

consequence discrimination between tissues would most readily be possible if the tissues involved differed greatly in their constitution.

Certainly approaching the study of biological activities by the application of regular solution theory is not above severe criticism, especially if a chemically and biologically rigorous theoretical framework is desired. In attempting to apply this theory it is perhaps appropriate to take a dual perspective, the choice depending on the use that is to be made of the method. If the intention is to test the approach in terms of rigorous foundations, it would be best to consider only relatively nonpolar substances and their action on the most simple of tissues for which δ values may be determined by swelling, vapour pressure, or osmotic pressure measurements. The contribution made by the Flory–Huggins[40–43] size correction, due to the difference in molar volumes of drug and biophase components, might also be determined. This correction could be included in equations 10 and 11 as an additional set of terms given by

$$-RT\left[\ln \Phi_2 + \Phi_1\left(1 - \frac{V_2}{V_1}\right)\right] \tag{12}$$

From a drug design standpoint, however, a more flexible yet readily applied procedure is most desirable. In this case the molar volumes and solubility parameters in equations 10, 11 and 12 may be considered to be adjustable, the separate relationships providing an empirical framework within which to work. This latter view is adopted in this article.

An alternative criticism might take note that Ferguson's development applies only to an equilibrium situation and hence should not be carried over into kinetic situations, e.g., membrane penetration. That this criticism is invalid is easily demonstrated from a consideration of Fick's Law for diffusion through thin membranes, which is the usual model used in biological work. The steady-state rate of penetration (dQ/dt) of a drug through a membrane is most simply represented by the relation

$$\frac{dQ}{dt} = \frac{DM}{\Delta x} KS = kS \tag{13}$$

where K is the partition coefficient for distribution of a compound between the membrane and the applied vehicle, D is the effective average diffusivity of the drug in the membrane, M is the effective cross-sectional area through which the diffusion flux passes, and Δx is the thickness of the membrane. An alternative way of writing equation 13 is

$$\frac{dQ}{dt} = \frac{DM}{\Delta x} \frac{a_a}{\gamma_b} \tag{14}$$

in which a_a is the thermodynamic activity of the drug in its vehicle and γ_b is the effective activity coefficient of the agent in the membrane. Thus, taking the logarithm of an observed penetration constant leads to a modification of equation 10 in which the quantity $\log (DM/\Delta x)$ is a part of the intercept. Alternatively, since biological activities are usually taken after a fixed time interval, they may be taken as analogous to an instantaneous rate.

Hence, taking the logarithm of the biological response measure corresponds to $\log (dQ/dt)_t$ and again equations 10 and 11 are seen to apply.

Since, in taking a regular solution theory approach to analyzing biological data, attention is focused on the activity coefficient γ, it is appropriate to ask what the range in activity coefficients might be as it is transferred from one medium to another. What this range could be for biological tissues is as yet an open question, but extremely wide variations in the activity coefficient have been reported by Higuchi[44] for Sarin, a nerve gas, in differing solvents. *Table 3* reproduces these values to show the variation that has been observed.

Table 3. Limiting activity coefficients of Sarin in solvents[44].

Substance	γ
Perfluorotributylamine	66.6
Hexadecane	15.6
Water	14
Tributylamine	10.4
Tetralin	4.3
2-Pyrrolidone	2.8
Diethylene glycol	2.4
Carbon tetrachloride	2.4
Phenyl ether	2.38
Diisooctyl adipate	1.84
Methyl salicylate	1.74
N-Methylacetamide	1.44
Dibutyl phthalate	1.42
Butyrolactone	1.31
Isoamyl alcohol	1.07
Ethyl lactate	0.536
Benzyl alcohol	0.446
m-Cresol	0.044

APPLICATION TO STUDIES OF ERYTHROCYTE HAEMOLYSIS

A simple membrane for which an extensive literature exists and which has been the subject of a recent extrathermodynamic study[45] is the erthrocyte membrane. This system was used by Roth and Seeman[28, 29] in verifying the Meyer hypothesis and as a consequence should be capable of analysis in terms of regular solution theory. We show here that (a) data correlating linearly with *n*-octanol:water partition coefficients sometimes show a definite parabolic trend when plotted against solubility parameters. More frequently, when such comparisons are possible, there is general agreement between the type of curve obtained by either approach, i.e., linearity in one case also is found with the other or quadratic behaviour with one is also found with the other. (b) Identical conclusions are obtained taking either approach regarding the similarity in the nature of the erythrocyte membrane in differing animal species. (c) For the erythrocyte membrane, equation 11, and not equation 10, is the more suitable basis for interpretation, since the

coefficients to the derived regression equations are related in a more consistent manner with equation 10 than with equation 11. (d) The apparent δ value for erythrocyte membrane seems to be about 8.08, which can be contrasted with an apparent δ value of around 10.5 reported[6, 46] for nerve membrane.

Solubility parameters calculated[47] for the compounds compiled by Hansch and Glave[45] when plotted with their haemolytic concentrations as the dependent variable tended to provide curves paralleling the type of equation reported from regression analysis. *Figures 1a* and *1b* show this

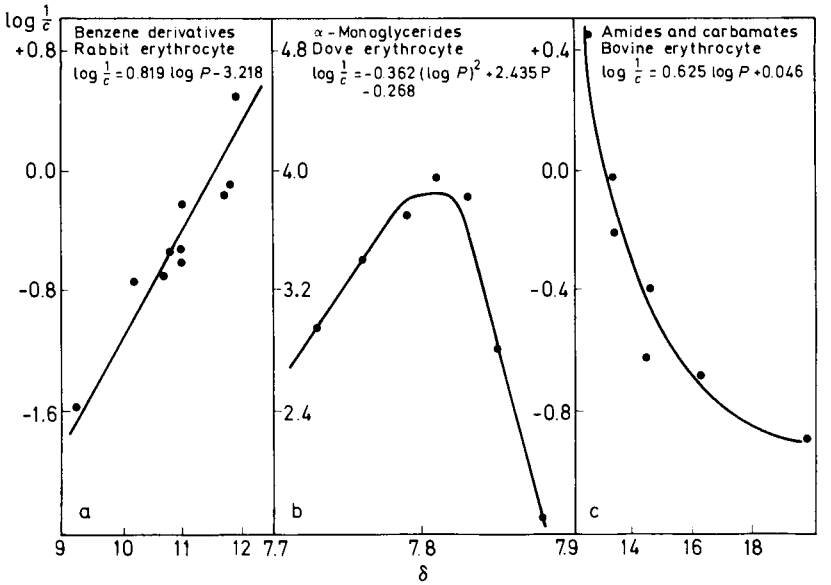

Figure 1. Comparison of haemolytic activities correlated with partition coefficients and plotted against the solubility parameter δ.

correspondence for substituted benzenes tested against rabbit erythrocyte and α-monoglycerides tested against dove erythrocyte. In some instances, however, as for amides and carbamates tested against bovine erythrocyte (*Figure 1c*), curvature in the solubility parameter plot is noted while the equation involving partition coefficients was reported to be linear. While nothing substantive can be made of this observation, the discrepancy between the two types of correlative approaches might be interpreted as indicating that a distinction between a simple partitioning model and a saturation model could be made using erythrocyte membrane data.

As may have been expected, not all the data compiled by Hansch and Glave[45] led to smooth curves when δ values were used as a measure of solubility. The reasons for this are not entirely clear, although it should be pointed out that for some sets of data there were uncertainties as to whether good approximations to F and V were used in calculating the δ values, while

Table 4. Regression equations for erythrocyte membrane haemolysis derived for the model $\log (1/C) = a + b\delta + c\delta^2$

Erythrocyte source	Type of compound	a	b	c	Temperature, °C	δ_n or δ^{opt}
Human	$ROSO_3^- Na^+$	1482	−408.1	27.99	24	7.28
	$RNMe_3^+ Br^-$	3726	−997.4	66.66	37	7.48
Bovine	ROH	718.4	−162.4	9.163	21	8.85
	RCO_2R	210.6	−45.20	2.423	21	9.32
Rabbit	ROH	650.9	−146.8	8.272	18	8.87
	$PhCH_2NR_3^+ Cl^-$	−8497	2152	−136.2	37	7.90
Dog	RCO_2H	1343	−362.4	24.48	25	7.40
	$RNH_3^+ Cl^-$	3679	−968.5	63·74	30	7.59
	N-R-pyridinium I^-	3385	−890.9	58.62	30	7.59
Sheep	$RCHBrCO_2^- K^+$	2465	−644.4	42.15	37	7.64
	$RNMe_3^+ Br^-$	−3692	913.6	−56.41	37	8.09
	$ROSO_3^- Na^+$	−5687	1432	−90.15	37	7.94
	$PhCH_2NMe_2R^+ Cl^-$	−2297	565.8	−34.73	37	8.13
Dove	RCO_2H	−4624	1170	−73.99	38	7.90
	α-R-monoglycerides	−15806	4058	−260.4	37	7.79
	N-R-piperidine Cl^-	−3014	754.5	−47.13	37	8.00
	N-R-pyridinium Br^-	−7436	1870	−117.5	37	7.95

with other sets it seemed possible to group compounds into subsets based on chemical structure. Of course another possibility is that it is hoping for too much to have regular solution theory apply to all the compounds. Those sets of data which provided smooth curves of a definite quadratic nature were thus selected for regression analysis making use of an orthogonal polynomials routine for the curve fit. *Table 4* summarizes the equations that have been derived in this manner[47].

It will be noted from *Table 4* that the signs to the coefficients are reversed in some instances but not in others. This may be recognized to depend on whether the compounds function in a disruptive or a protective manner towards erythrocytes. Since one action is the reverse of the other, the equations referring to, say, antihaemolytics should be multiplied through by -1. The parabola is thus defined always to be concave with respect to the x-axis, and the coefficients can then be compared in the same relative manner.

The question now arising is whether the equations represented in *Table 4* are best interpreted in terms of a distribution model (equation 10) or a saturation model (equation 11). By equating corresponding terms of equation 10 with the coefficients of a relationship from *Table 4*, making what seemed to be reasonable assumptions for the δ value of water ($\delta = 23$[4] and 16[48,49]) and the molar volumes for the compounds in the membrane (taken to be the same as the pure compound), and solving the simultaneous equations that were obtained, it was not possible to arrive at a δ value for the membrane that was positive in sign. It was thus concluded that a distribution model for erythrocyte haemolysis is most probably invalid.

The corresponding analysis involving equation 11 was approached in a different manner. Since the equilibrium concentration of drug in the erythrocyte membrane is expected[28,29] to be low it was presumed that the term involving S_b in equation 11 could be neglected. Differentiation of equation 11 with respect to the δ value for drug shows that the maximum haemolytic effect occurs at an optimum solubility parameter value δ^{opt} and this value is the same as that for the membrane δ_m. To determine δ^{opt} from the regression coefficients, it is thus necessary to use the relationship

$$\delta^{opt} = b/2c \tag{15}$$

However, from inspection of equation 11 it can be noted that the square root of the regression coefficient ratio a/c should provide an alternative estimate of the δ value for the membrane

$$\delta_m = (a/c)^{\frac{1}{2}} \tag{16}$$

Interpretation of the regression coefficients found in *Table 4* according to equations 15 and 16 leads to excellent agreement between the calculated values for δ^{opt} and δ_m. Membrane apparent solubility parameters appropriate to each data set are listed in *Table 4*. These average to 8.08 which should be contrasted with a value of about 10.5 reported[6,46] for nerve membrane. It may thus be stated that erythrocyte haemolysis involves a saturation-type process and not a distribution-type process. This conclusion is fully compatible with the saturation-like sigmoid curves frequently observed in plots of percentage haemolysis *versus* drug concentration, as shown for example by *Figure 2*. A similar extension to this type of experiment is not

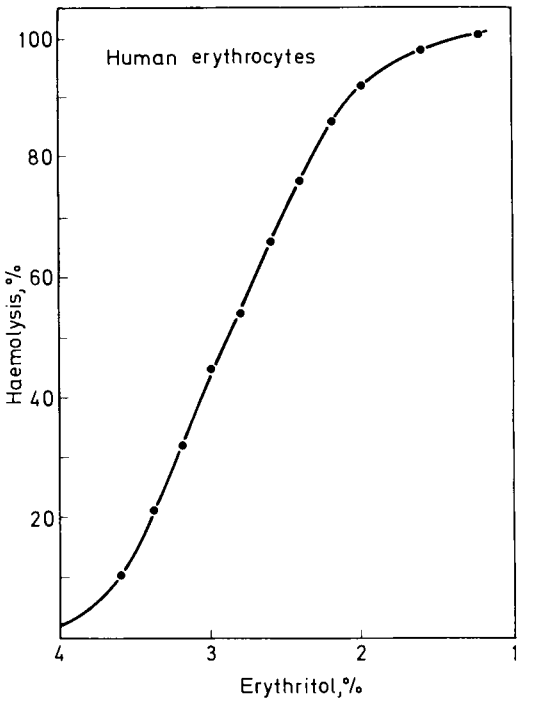

Figure 2. Typical percentage haemolysis *versus* dose of drug curve. (P. Zanowiak, Ph.D. Thesis, University of Florida, Gainseville, Florida, 1959)

indicated in any way from the results of an extrathermodynamic type of approach.

Having found regular solution theory to apply to a number of studies of erythrocyte haemolysis, it is pertinent to inquire into the possible reasons for this apparent success. At first sight, the compounds shown in *Table 4* would not appear suitable for study by regular solution theory, since they are either ionic or contain a functionality capable of hydrogen bonding. A plausible rationale for the findings takes note of the behaviour of surface active agents at an organic medium–aqueous medium interface. Surface active agents possess a polar or charged moiety attached to an alkyl group and, at an organic–aqueous interface, the polar groups tend to be associated with the aqueous phase while the nonpolar groups tend to be in the organic phase. For erythrocyte haemolysis, the polar components of the compounds found in *Table 4* would be associated with the aqueous assay medium while the nonpolar groups would be contained in the lipophilic interior of the membrane. Presuming the haemolytic action of the compounds to be a consequence of disruption of the interior organization of the membrane by the nonpolar substituent, it would thus be reasonable to expect regular solution theory to be applicable to erythrocyte haemolysis. The polar group no doubt also contributes to disruption of the membrane, but this

functionality is maintained constant with each series of compounds and whatever contribution made by it relative to the nonpolar fragment may be considered relatively invariant in passing from one compound to another.

CONCLUSIONS

A number of differing types of biological activities, such as narcotic potency, enzymatic activities, and protein binding, have been correlated against electronic polarizability[50–54] or, its magnetic equivalent, diamagnetic susceptibility[55]. It has recently been shown[39] that these electronic indexes can be used to calculate molar attraction constants, and hence δ values, in a semi-empirical manner for nonpolar and slightly polar molecules. The correlations of biological activity with electronic polarizability may thus be said to have a basis in regular solution theory[10]. Of much more significance, however, is the attempt by Davis[56] to calculate the extrathermodynamic lipophilic parameter π using solubility parameters and molar volumes. An encouraging degree of agreement was obtained between the calculated and observed values, prompting us[57] to seek a correlation between partition coefficients and electronic polarizability. It can be stated that at least for the *n*-octanol–water distribution system a surprisingly good correlation between partition coefficient and electronic polarizability is found[57], the correlation extending over 72 compounds. Two lines are found, one for the compounds with electronegative atoms (N, O) and the other for compounds that are ordinarily considered relatively nonpolar. The two lines suggest two types of partitioning processes which are potentially explicable in terms of molal volume differences for a compound contained in two differing phases[10]. The latter correlation thus suggests that a large proportion of the relationships between biological activities and partition coefficients[1–3] may be profitably reinvestigated in terms appropriate to regular solution theory. The results of our[55, 57] investigations along these lines will be reported in the near future.

REFERENCES

[1] C. Hansch. In *Physicochemical Aspects of Drug Action*, (E. J. Ariens, Ed.), Pergamon, London (1968).
[2] C. Hansch, *Accts. Chem. Res.* **2**, 232 (1969).
[3] C. Hansch. In *Drug Design*, (E. J. Ariens, Ed.), Academic, New York (1971).
[4] J. H. Hildebrand and R. L. Scott, *The Solubility of Nonelectrolytes*, 3rd Edition, Dover, New York (1964).
[5] J. H. Hildebrand, J. M. Prausnitz, and R. L. Scott, *Regular and Related Solutions*, Van Nostrand Reinhold, New York (1970).
[6] L. J. Mullins, *Chem. Rev.* **45**, 289 (1954).
[7] L. J. Mullins, *Ann. N. Y. Acad. Sci.* **62**, 247 (1955).
[8] K. W. Miller, W. D. M. Paton, and E. B. Smith, *Nature*, **206**, 574 (1965).
[9] J. A. Ostrenga, *J. Med. Chem.* **12**, 349 (1969).
[10] A. Cammarata, S. J. Yau, and K. S. Rogers, *J. Med. Chem.* **14**, 1211 (1971).
[11] W. E. Moore, *J. Amer. Pharm. Assoc., Sci. Ed.* **47**, 855 (1958).
[12] M. J. Chertkoff and A. N. Martin, *J. Amer. Pharm. Assoc., Sci. Ed.* **49**, 444 (1960).
[13] W. E. Moore, *J. Pharm. Sci.* **51**, 391 (1962).
[14] F. A. Restaino and A. N. Martin, *J. Pharm. Sci.* **53**, 636 (1964).
[15] W. G. Gorman and G. D. Hall, *J. Pharm. Sci.* **53**, 1017 (1964).

[16] S. A. Khalil and A. N. Martin, *J. Pharm. Sci.*, **56**, 1225 (1967).
[17] M. A. Augustine and J. Swarbrick, *J. Pharm. Sci.*. **59**, 314 (1970).
[18] A. P. Shroff and C. J. Shaw, *Can. J. Pharm. Sci.* **6**, 24 (1971).
[19] A. N. Paruta, B. J. Sciarrone, and N. G. Lordi, *J. Pharm. Sci.* **51**, 704 (1962).
[20] A. Leo, C. Hansch, and C. Church, *J. Med. Chem.* **12**, 766 (1969).
[21] A. Leo, C. Hansch, and D. Elkins, *Chem. Rev.* **71**, 525 (1971).
[22] A. Leo and C. Hansch, *J. Org. Chem.* **36**, 1539 (1971).
[23] C. Richet, *Compt. Rend. Soc. Biol.* **45**, 775 (1893).
[24] E. Overton, *Viertljahrsschr. Naturforsch. Ges. Zurich*, **44**, 88 (1899).
[25] H. Meyer, *Arch. Exptl. Pathol. Pharmakol.* **42**, 109 (1899).
[26] R. Collander, *Trans. Faraday Soc.* **33**, 985 (1937).
[27] K. H. Meyer, *Trans. Faraday Soc.* **33**, 1063 (1937).
[28] H. Machleidt, S. Roth, and P. Seeman, *Biochim. Biophys. Acta*, **255**, 178 (1972).
[29] S. Roth and P. Seeman, *Biochim. Biophys. Acta*, **255** 207 (1972).
[30] J. Ferguson, *Proc. Roy. Soc. (B)*, **127**, 387 (1939).
[31] E. T. Burtt, *Ann. Appl. Biol.* **32**, 247 (1945).
[32] F. Brink and J. M. Pasternak, *J. Cellular Comp. Physiol.* **32**, 211 (1948).
[33] N. A. Allawala and S. Riegelman, *J. Amer. Pharm. Assoc., Sci. Ed.* **42**, 267 (1953).
[34] N. A. Allawala and S. Riegelman, *J. Amer. Pharm. Assoc., Sci. Ed.* **43**, 93 (1954).
[35] J. Ferguson and H. Pirie, *Ann. Appl. Biol.* **35**, 532 (1948).
[36] J. Ferguson and S. W. Hawkins, *Nature*, **164**, 963 (1949).
[37] P. A. Small, *J. Appl. Chem.* **3**, 71 (1953).
[38] J. A. Ostrenga, *J. Pharm. Sci.* **58**, 1281 (1969).
[39] A. Cammarata and S. J. Yau, *J. Pharm. Sci.* **61**, 723 (1972).
[40] P. J. Flory, *J. Chem. Phys.* **9**, 660 (1941).
[41] P. J. Flory, *J. Chem. Phys.* **10**, 51 (1942).
[42] M. L. Huggins, *J. Chem. Phys.* **9**, 440 (1941).
[43] M. L. Huggins, *Ann. N. Y. Acad. Sci.* **43**, 1 (1942).
[44] T. Higuchi, *J. Soc. Cosmetic Chem.* **11**, 85 (1960).
[45] C. Hansch and W. R. Glave, *Mol. Pharmacol.* **7**, 337 (1971).
[46] S. Dikstein. In *Quantitative Methods in Pharmacology*, (H. Dejonge, ed.), North-Holland, Amsterdam (1961).
[47] A. Cammarata and S. J. Yau, unpublished results.
[48] A. Koskas and J. Derandet, *Chem. Ind.* **98**, 1386 (1967).
[49] T. Wakahayashi, S. Oki, T. Omori, and N. Suzuki, *J. Inorg. Nucl. Chem.* **25**, 1351 (1963).
[50] L. Pauling and D. Pressman, *J. Amer. Chem. Soc.* **67**, 1003 (1945).
[51] J. A. Clements and K. M. Wilson, *Proc. Natl. Acad. Sci., U.S.* **48**, 1008 (1962).
[52] D. Agin, L. Hersh, and D. Holtzman, *Proc. Natl. Acad. Sci., U.S.*, **53**, 952 (1965).
[53] C. Coats, W. R. Glave, and C. Hansch, *J. Med. Chem.* **13**, 913 (1970).
[54] A. Cammarata, *J. Med. Chem.* **10**, 525 (1967).
[55] A. Cammarata, S. J. Yau, and K. S. Rogers, unpublished results.
[56] S. S. Davis, *Experientia*, **26**, 671 (1970).
[57] A. Cammarata and K. S. Rogers, unpublished results.

THE PREDICTION OF MOLECULAR CONFORMATION AS A BIOLOGICALLY SIGNIFICANT PROPERTY

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ABSTRACT

The value of molecular orbital theory in the hands of the chemist or biologist lies, hopefully, in its ability to derive information concerning the properties of molecules of interest to him, without complete reliance on the performance of an experiment. This expectation is becoming more of a reality as a result of work using all-valence electron semi-empirical molecular orbital methods to study conformations of drug molecules. Some of the work in our laboratory is described with notations as to the general agreement with experiment. The frequent insight into the pharmacophore definition is a major contribution of these studies. Finally, the implication for explanations of drug mechanisms and new drug design is present in each topic discussed.

INTRODUCTION

Chemical and physical events involving a drug at its critical point of action in the body are, at this time, obscure. We assume a specific target for many drug molecules and term this a receptor. If we acknowledge that a drug must be absorbed, transported, penetrate barriers and survive metabolism, we are left with the conclusion that rather specific structural features are necessary for a drug to efficaciously engage a receptor. These structural characteristics are basically (a) those atomic features suitable for the requisite drug–receptor interaction phenomena and (b) the appropriate spatial disposition of these features necessary to bring about the required simultaneous or required sequential interaction events with the receptor.

Since insight into the intricacies of receptor structure are currently denied us, we must examine molecules of high potency and therefore presumably of optimal interaction with receptors, to gain insight into the drug–receptor phenomena. If we can define what is essential in a molecule for optimal receptor interaction we can generalize as to what that receptor requires in a molecule (pharmacophore). We may further speculate on what may be the complementary binding features of a receptor. The generalization of a pharmacophore provides us with some rationale for the design of agonists and competitive antagonists.

The definition of electronic properties of a molecule, such as electron distribution, can frequently be accomplished in an approximate way from

intuition. A knowledge of relative electronegativities of atoms in a molecule permits us to make relative assignments of charge excess or charge deficiency, in many cases.

A more complex problem arises when the identified pharmacophore is embedded in a molecule in which we cannot intuitively predict the shape or conformation. A very large number of drugs and agonists are molecules with at least one sigma bond and hence with at least one degree of doubt as to the structure in space. Indeed the estimation of conformation in many cases becomes the limiting factor separating us from a useful definition of a molecular structure.

We have known for some years that sigma or single bonds are neither free to rotate nor are they entirely rigid. The composition of conformational isomers at equilibrium, and the associated energy barriers are governed by a summation of all attractions and repulsions within the molecule. This mixture of patterns must then prevail in drug molecules in the vicinity of a receptor. How then can we hope to define what is the 'active' conformation at a receptor?

Over the years we have conducted our studies on drug molecule conformations with the operational hypothesis that the energetically preferred conformation was the 'active' conformation at the receptor[1, 2]. This hypothesis has obviously been adopted by many investigators both before and since our earliest work. They have adopted the hypothesis since they have sought by various means to define what the preferred conformation is of many drug molecules, and have used their findings to explain or predict biological events.

It is very likely that intimate interaction between a drug and a receptor will mutually perturb both molecules so that new conformations will dominate, governed by the energetics of the new environment. It is our basic hypothesis however that at the most remote distance of drug–receptor engagement it is the preferred conformation of the drug or a conformation very close in energy to the preferred conformation which is 'recognized' by the receptor. If the correct pharmacophore is present in the molecule at the time of 'recognition' then upon closer approach, the appropriate interactions will occur leading to an efficacious biological event. As a net gain in energy of interaction or bonding occurs, mutual perturbation will alter both species along energy-determined pathways, leading to either an efficacious effect if the pharmacophore is present or to an inactive affinity. This then is the hypothesis of *remote recognition of preferred conformation.*

We have found in several of our studies that when more than one potent agonist in a pharmacological class is analysed for preferred conformation, there are predicted or observed nearly identical patterns of essential features in the preferred conformations. This certainly suggests more than a coincidence. It reinforces our belief that the preferred conformation is a highly relevant structural parameter contributing to the drug's action. We interpret this as supporting the hypothesis of *remote recognition of preferred conformation.*

EVALUATION OF CONFORMATION

The prediction of the conformation of molecules has been of considerable interest to biological scientists for many years. Early approaches centred on

an intuition based on the presumed repulsive interaction of bulky groups across space. This gave rise to general rules of conformational preference which had some utility with small molecules. Unfortunately, this intuition was unable to perceive the attractive forces which are also a part of the total influence on conformation. Attractive forces are particularly prominent in heteroatom molecules and these are predominantly what the biological scientist encounters.

The advent of x-ray crystallography has permitted the mapping of the atoms of molecules in the solid state. The relevance of these conformations to solution phenomena is, however, obscure. In the crystal, the molecules are closely packed, interacting with each other and with gegenions if present. This is probably not the situation normally encountered in the dilute solutions of the biological milieux. Thus, biological conclusions derived from conformations deduced from x-ray crystallographic analyses must always be considered in this light.

A more useful experimental approach to predicting conformation in a biological environment is through the use of n.m.r. analysis in water. This data, if properly analysed, gives a time-average conformation which can be of considerable value in subsequent biological interpretations. It is necessary, however, to actually have the compound under study and frequently the analysis of the n.m.r. data is extremely complex.

Other solution techniques for predicting preferred conformation include o.r.d., dipole movement and spectroscopic methods. Each is capable of giving useful, partial information on molecular conformation. Each, of course, requires that the compound be actually available for study.

MOLECULAR ORBITAL PREDICTION OF CONFORMATION

Another approach which has become available in the past decade is the use of all-valence electron, semi-empirical molecular orbital theory. This approximation of quantum mechanics makes it possible to calculate for fairly large molecules, a total energy behaving in an approximately parallel fashion to .the true molecular energy. The consideration of all valence electrons makes this calculated total energy sensitive to the conformation of the molecule. Thus, energy minimization as a function of bond-angle variation is possible and the prediction of a preferred conformation is a consequence.

The first of these methods was developed by Hoffmann in 1963[3], and is known as extended Hückel theory (EHT). Briefly, the method employs Hückel formalism; however, explicit consideration of non-bonded interactions and all overlap integrals are a refinement. Slater orbitals are employed and the computations require only one parameter, the valence state ionization potential for the Coulomb integral and indirectly for the resonance integral. The theory, the merits and evaluations of EHT have been reported[4–6].

More recently, an all valence electron, semi-empirical molecular orbital theory known as the Complete Neglect of Differential Overlap (CNDO) has been proposed by Pople based on self-consistent field (SCF) formalism[7]. Although this method employs a more sophisticated approximation of the wave function, it neglects differential overlap.

Comparisons of these two methods reveal their relative strengths and shortcoming[4]. In general, the CNDO method is superior for charges, EHT predicting greatly exaggerated values. The major value of EHT lies in its ability to correctly predict the preferred conformation. This has been demonstrated for numerous hydrocarbons[3] and more recently for a wide variety of heteroatom molecules[1, 2].

A large number of molecular orbital predictions of biological molecule conformations have been accomplished using EHT. The record of agreement between calculated and experimental values has been good. A significant amount of useful information has emerged from these predictions pertinent to the structure–activity relationships, the consideration of molecular mechanisms, and the rationale for new drug design[1, 2].

MUSCARINIC AGENTS

The first MO study of the conformation of a neurotransmitter was reported on acetylcholine[8]. The prediction of a *gauche* chain is in agreement with an n.m.r. analysis[9]. In the same study, the prediction of the conformations of the muscarinic agonists muscarine and muscarone were reported. Both predictions have been supported by x-ray analyses[10, 11]. This early work led to the encouraging belief that EHT calculations of conformation could mirror physical reality. The study also revealed that all three muscarinic agonists in their predicted preferred conformations resulted in the virtual superpositioning of the heteroatoms. This led to the prediction that this pattern of the heteroatoms was the muscarinic pharmacophore, *Figure 1*. It is noteworthy that this pharmacophore is practically identical to the muscarinic pharmacophore derived from extensive structure activity

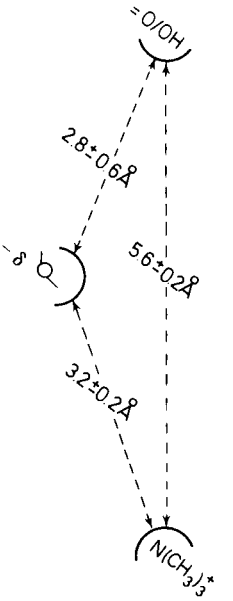

Figure 1. Predicted muscarinic pharmacophore[8]

studies[12]. The findings support the hypothesis that the preferred conformation is relevant to this series of drug–receptor events.

Similar calculations on oxotremorine, a CNS muscarine agent, revealed a pattern of structural features compatable with *Figure 1*[13].

The early success of these studies both in their agreement with experiment and in the biological significance of the interpretations, have resulted in the continued use of this method and general approach[1, 2].

NICOTINIC AGENTS

In a companion study to the muscarinic agents, calculations were performed on the nicotinic agent nicotine[14]. Two equivalent conformations were found in agreement with n.m.r. data[15]. In one of these conformers there was found a distance separating the pyridyl nitrogen and onium group, comparable to the carbonyl oxygen–onium group distance in a conformer of acetylcholine. This pattern was proposed as the nicotinic pharmacophore,

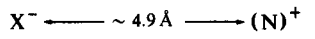

Figure 2. Predicted nicotinic pharmacophore[14]

Figure 2 and that conformer of acetylcholine proposed as being the nicotinic form of acetylcholine[14]. MO-calculated conformations of other potent nicotinic agents such as phenylcholine ether[16] and neostigmine[17] support this pharmacophore hypothesis. The prediction of the phenylcholine ether conformation is in agreement with the crystal conformation of xylocholine[18].

As a result of these studies, the conclusion was reached that acetylcholine exerts two actions, muscarinic and nicotinic, by engaging two different receptors with two different pharmacophores. The two pharmacophores are constituted from different sets of functional features in different conformations of the molecule[2].

HISTAMINE

Molecular orbital calculations have led to the prediction of two equivalent conformations of histamine with *gauche* and *trans* side chains respectively[19].

This prediction has been verified by n.m.r. analysis[20]. A CNDO-type calculation predicts only a *gauche* conformation as preferred[21].

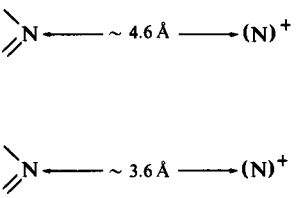

Figure 3. Predicted histamine pharmacophores[19]

On the basis of the prediction of the two conformations, the hypothesis was proposed that the dual action of histamine was due to the engagement of one receptor, designated H_1, with the *trans* conformer and the second receptor, the acid secretory receptor H_2, with the *gauche* conformer[19], *Figure 3*. This is then a parallel of the acetylcholine dual-action, dual-conformation hypothesis[2, 8, 14].

SEROTONIN

The preferred conformation of serotonin was predicted, using EHT, to have a *trans* side chain[22]. Modified CNDO[23] and INDO[24] calculations have predicted a *gauche* to a *cis* preference for the side chain. Recent n.m.r. analysis reveals a *trans* preference[25], in agreement with the EHT calculation.

A serotonergic pharmacophore was postulated on the basis of the predicted conformation[22], *Figure 4*. It is interesting to note that the inter-nitrogen

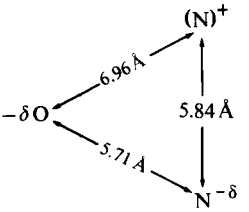

Figure 4. Predicted serotonin pharmacophore[22]

distance in this pharmacophore is mimicked in the lysergic acid diethylamide (LSD) molecule, a competitive antagonist. Another similarity has been commented upon, relating the serotonin pharmacophore and the active metabolite, 11-hydroxy-Δ^9-tetrahydrocannabinol[26]. This could be a significant relationship in the hallucinogenic action of this molecule and LSD.

PREGNANE STEROIDS

The side chain conformations of progesterone, corticosterone and cortisol were predicted using EHT–MO[27]. All three side chains were predicted to form a plane with the 17α substituent. The prediction of the progesterone side chain conformation is within 30° of a solution dipole moment study[28]. The predictions of the corticosterone and cortisol conformations are in close agreement with experimental results from infrared and n.m.r. studies[29, 30].

Portions of the predicted cortisol pattern of charged atoms, *Figure 5*, were observed to be comparable to charged patterns for either histamine[19] or serotonin[22]. Since these two amines have been implicated as being inflammagenic[31] and cortisol is a potent anti-flammatory agent[32], it was postulated that cortisol might evoke this action by an interaction with either or both histamine and serotonin receptors by virtue of these common structural features.

The hypothesis has received some experimental support from the recent observation that cortisol is effective in competing for histamine-binding sites

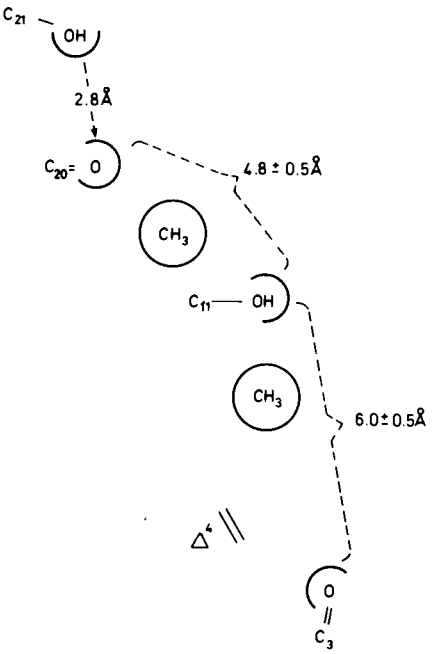

Figure 5. Predicted cortisol pattern[27]

on biopolymers[33]. A subsequent EHT–MO study involving non-steroidal anti-inflammatory agents including indomethacin confirmed that the common structural patterns predicted, prevailed in these drugs[34]. The predicted conformation of indomethacin is in agreement with the reported crystal structure of this molecule[35]. It is interesting to contemplate the roles of serotonin as a platelet aggregation promoter and the common anti-inflammatory agents as platelet aggregation inhibitors as a possible parallel of this hypothesis of anti-inflammatory activity.

ADRENERGIC AGENTS

EHT–MO predictions of the adrenergic agents ephedrine and pseudoephedrine have been reported[36]. The conformation predicted for ephedrine is in agreement with n.m.r. analysis[37, 38]; however, the prediction of the pseudoephedrine conformation agrees only with a minor contributor to the solution equilibrium[38]. The predicted conformation for the α-adrenergic agonist, norepinephrine, presents the same pharmacophore[39], *Figure 6*. This prediction is in agreement with the crystal conformation[40]. Other studies using the INDO[41] and CNDO methods[42] predict the coexistence of a *trans* and a *gauche* conformation.

It is well known that an unsubstituted catecholamine such as norepinephrine or a monomethyl derivative like epinephrine is predominantly α-adrenergic. Increasing the bulk of the mono-alkyl substituent in this series

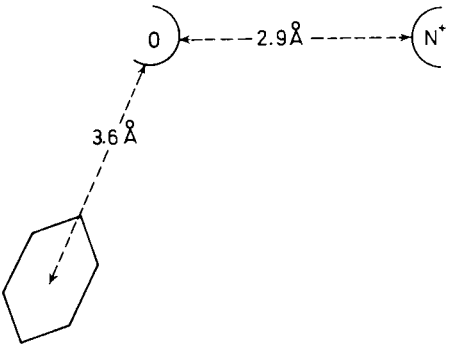

Figure 6. Predicted α-adrenergic pharmacophore[36, 39]

increases β-adrenergic activity while at the same time α-adrenergic activity is obliterated. Thus, isopropyl norepinephrine (isoproteranol) is a standard for almost pure β-adrenergic activity.

Two theories have been proposed to explain this reversing pair of trends in the catecholamine series. One theory proposed that the increasing bulk of the N-substituent increases the barrier to rotation between the methylene groups so that the ease of assuming a *gauche* conformation will be a function of the N-substituent bulk[43]. The theory then proposes that the different conformers, *gauche* and *trans*, influence the occurrence of two different reactions, each characteristic of the α and β adrenergic receptor. The second theory proposes that the N-substituent influences the charge on the onium group which influences the reactivity to one or the other adrenergic receptor[44].

A recent study employing EHT for conformation and CNDO and *ab initio* calculations for charge densities has been reported on the catecholamine series norepinephrine, epinephrine, *N*-ethylnorepinephrine and isoproteranol[45]. These studies revealed no predicted change in the *trans* preference for any of the series. Further, they showed an almost identical energy of the barrier from a *trans* to a *gauche* conformation for all members of the series. These results argue against the theory of variable flexibility of the methylene–methylene bond[43]. Charge densities were calculated on simulated onium systems using both CNDO and *ab initio* methods[45]. These results revealed no appreciable change in onium charge in the series, which is at variance with the charge theory[44].

An alternative theory was proposed based on these calculations. It was postulated that α-adrenergic activity requires an onium hydrogen atom, probably as a hydrogen bond donor. The β-adrenergic receptor, however, was postulated to require an alkyl group at the N-substituent position. It was proposed that this N-substituent was involved in a dispersion interaction with the receptor and was optimal when there was a branched hydrocarbon such as an isopropyl group. These authors raised the intriguing suggestion that the onium group has a constant influence or was perhaps not essential for β-adrenergic activity and that a methylene group could replace it. It was noted that such a compound has recently been made and reported[46]. This

compound, the methylene analogue of isoproteranol, was found to have modest β-adrenergic activity. A parallel was also drawn between this hypothesis and the structure and β-adrenergic activity of prostaglandin E_1[45].

DOPAMINE

An EHT calculation on dopamine, holding the ring hydroxyls out of the ring plane predicted a *gauche* conformation[47]. A calculation apparently on just the side chain conformation predicted a *trans* conformation[48]. A recalculation of the molecule, holding the hydroxyls in the ring plane, predicts a *trans* conformation[26]. Crystal studies indicate a *trans* preference for the salt[49] while n.m.r. analysis indicates a contribution from both isomers[48]. It has been suggested that the *gauche* conformer of dopamine might account for its ability to interact with a receptor different from that of noradrenalin[47].

An interesting structural similarity can be observed between dopamine and the morphine-like analgesics. A basic nitrogen atom can be found, in all of these compounds, about 4 Å from the centre of a phenyl ring. This is a dimension found in the *gauche* form of dopamine[47]. It is possible that the mechanism of analgesia involves dopamine with the analgesics acting as inhibitors at a specific site.

CENTRAL INHIBITORY TRANSMITTERS

Calculations on the conformation of γ-aminobutyric acid (GABA) and an agonist, muscimol, have revealed a common pattern of charged structural features arising from a *trans* conformation of GABA, leading to the prediction of a pharmacophore[50]. Support for this prediction can be found in the fact that the structurally rigid acetylenic analogue aminotetrolic acid is a GABA agonist[51].

Further support is found in the prediction of a conformation for the competitive antagonist bicuculline, in which the hypothetical pharmacophore for GABA is reproduced[52]. Calculations on the agonist β-hydroxy GABA indicate a *gauche* and a *trans* preference[52].

In contrast to GABA, the centrally-acting inhibitory transmitter glycine is predicted to have a conformation not resembling GABA in its salient structural features[52]. This supports the belief that these two agents are acting at separate receptors.

PROSTAGLANDIN

Prostaglandin E-1 has recently been studied using EHT for some conformational properties, iterative EHT for atom charges and a method using monopole-bond polarizabilities for the calculation of the interaction energies between the side chains, contributing to the conformation[53]. The calculations predicted a graded series of preferences for the side chains, the most prominent of which involve an intimate interaction and mutual fixation of the side chain.

The dispersion forces are significant, exceeding 2 kcal $mole^{-1}$ in the most preferred conformer. These predictions are consistent with a recent x-ray

study which reveals one of the most prominent of the predicted conformers[54]. Structural rigidity imparted by the side chain interactions thus appears to be an essential feature in these molecules. These predictions may afford a means of studying the influence of side chain modifications and their influence on the diverse actions of these molecules.

PEPTIDE HORMONES

Following the initial predictions of amino acid conformations using EHT in 1969[55–57] a number of studies have been reported on all-valence electron MO methods in this area (recently reviewed[26]). There is general agreement found between prediction and available experimental evidence. Two studies have been reported on the conformations of small polypeptide hormones. These have been predicted from the individual residue predictions for bradykinin[58] and gastrin[59]. Hopefully these studies can point the way toward the very desirable goal of predicting small polypeptide conformation.

SWEET TASTE PHARMACOPHORE

At the present time it is believed that structural features imparting a sweet taste to a molecule include an atom capable of accepting a hydrogen bond (B) and a structure feature with a polar hydrogen (A—H), *Figure* 7[60]. The presence of this duo, A—H and B, can be found in a widely diverse group of

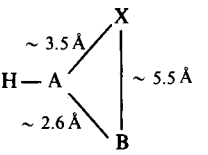

Figure 7. Predicted sweet taste pharmacophore or glucophore[62]

sweet-tasting molecules. A number of observations, however, suggest that this pharmacophore (or glucophore) may be incomplete. The fact that several D- amino acids are sweet while their L- isomers are bitter[61] is evidence of stereoselectivity, and a third receptor binding site.

EHT calculations on these amino acids has identified a common third molecular feature, assuming the A—H, B system to be N—H and an oxygen atom[62]. This has led to the postulation of a glucophore, *Figure* 7, with the third feature being an atom or group capable of dispersion bonding to a receptor feature.

Examination of a wide variety of sweet molecules of high potency has revealed the presence of this glucophore. Further evidence for dispersion bonding being involved at the third site of the glucophore is furnished by the finding of a good correlation between a group polarizability parameter and the sweetness level in a series of substituted nitroanilines[62].

CONCLUSION

With the passage of time and the contributions from our laboratory and others, we are strengthened in our view that MO theory can make a contribution to our understanding of drug molecule structure.

If the investigator is constantly aware of the nature of his semi-empirical methods, their strengths and limitations he can be guided into meaningful utilization of these methods. If he fully acquaints himself with the nature of biological events and how theory can explain or predict these events, meaningful applications can be made.

From the predictions made in our studies and the structure–activity hypotheses drawn, the medicinal chemist and the molecular pharmacologist have perhaps a new rationale for compound design or new relationships to derive mechanisms.

The validity of these hypotheses await further support. However, as is usually the case in scientific endeavour, the real scarcity is in new ideas. We feel that molecular orbital theory is a rich source for their derivation.

REFERENCES

[1] L. B. Kier, *Molecular Orbital Theory in Drug Research*, Academic Press, New York (1971).
[2] L. B. Kier in *Fundamental Concepts in Drug–Receptor Interactions* (J. F. Danielli, J. F. Moran and D. J. Triggle, eds.) Academic Press, New York (1970).
[3] R. Hoffman, *J. Chem. Phys.* **39**, 1397 (1963).
[4] J. R. Hoyland in *Molecular Orbital Studies in Chemical Pharmacology*, (L. B. Kier, ed.) Springer-Verlag, New York (1970).
[5] L. C. Allen and J. D. Russell, *J. Chem. Phys.* **46**, 1029 (1967).
[6] G. Blyholder and C. A. Coulson, *Theoret. Chim. Acta* **40**, 316 (1968).
[7] J. A. Pople, D. P. Santry and G. A. Segal, *J. Chem. Phys.* **43**, 5129 (1965).
[8] L. B. Kier, *Mol. Pharmacol.* **3**, 487 (1967).
[9] C. Culvenor and N. Ham, *Chem. Commun.* 537 (1966).
[10] F. Jellinek, *Acta Cryst.* **10**, 277 (1957).
[11] P. Pauling and T. J. Petcher, *Nature New Biol.* **236**, 112 (1972).
[12] A. H. Beckett, N. J. Harper and J. W. Clitherow, *J. Pharm. Parmacol.* **15**, 362 (1963).
[13] L. B. Kier, *J. Pharm. Sci.* **59**, 112 (1970).
[14] L. B. Kier, *Mol. Pharmacol.* **4**, 70 (1968).
[15] T. R. Simpson, J. C. Craig and W. D. Kumler, *J. Pharm. Sci.* **56**, 708 (1967).
[16] L. B. Kier, *J. Med. Chem.* **14**, 80 (1971).
[17] J. W. Crow and W. C. Holland, *J. Med. Chem.* **15**, 429 (1972).
[18] P. Coggon, A. T. McPhail and A. M. Roe, *Nature*, **224**, 1200 (1969).
[19] L. B. Kier, *J. Med. Chem.* **11**, 441 (1968).
[20] A. F. Casy, R. R. Ison and N. S. Ham, *Chem. Commun.* 1343 (1970).
[21] J. L. Coubeils, P. Courriere and B. Pullman, *Compt. Rend. Acad. Sci., Paris* **272**, 1813 (1971).
[22] L. B. Kier, *J. Pharm. Sci.* **57**, 1188 (1968).
[23] P. Courriere, J. L. Coubeils and B. Pullman, *Compt. Rend. Acad. Sci., Paris*, **272**, 1697 (1971).
[24] S. Kang and M. H. Cho, *Theoret. Chim. Acta* **22**, 176 (1971).
[25] R. R. Ison, P. Partington and G. C. K. Roberts, *J. Pharm. Pharmacol.* **24**, 84 (1972).
[26] L. B. Kier in *Advances in Chemistry*, **114**, 278 (1972).
[27] L. B. Kier, *J. Med. Chem.* **11**, 915 (1968).
[28] N. L. Allinger and M. A. DaRooge, *J. Am. Chem. Soc.* **83**, 4256 (1961).
[29] W. G. Cole and D. H. Williams, *J. Chem. Soc.* 1849 (1968).
[30] W. G. Cole and D. H. Williams, *J. Chem. Soc.* 748 (1970).
[31] W. G. Spector and D. A. Willoughby, *Ann. N.Y. Acad. Sci.* **116**, 839 (1964).
[32] C. A. Winter, *Progr. Drug Res.* **10**, 139 (1966).
[33] C. Botre, M. Marchetti, M. Del Vecchio, C. Lionetti and A. Memoli, *J. Med. Chem.* **12**, 832 (1969).

[34] L. B. Kier and M. W. Whitehouse, *J. Pharm. Pharmacol.* **20**, 793 (1968).
[35] T. J. Kistenmachen and R. E. Marsh, *J. Am. Chem. Soc.* **94**, 1340 (1972).
[36] L. B. Kier, *J. Pharmacol. Exptl. Therap.* **164**, 75 (1968).
[37] J. B. Hyne, *Can. J. Chem.* **39**, 2536 (1961).
[38] P. Portoghese, *J. Med. Chem.* **10**, 1057 (1967).
[39] L. B. Kier, *J. Pharm. Pharmacol.* **21**, 93 (1969).
[40] D. Carlstrom and R. Bergin, *Acta Cryst.* **23**, 313 (1967).
[41] L. Pedersen, R. E. Hoskins and H. Cable, *J. Pharm. Pharmacol.* **23**, 216 (1971).
[42] B. Pullman, J. L. Coubeils, P. Courriere and J. P. Gervois, *J. Med. Chem.* **15**, 17 (1972).
[43] A. A. Larsen, *Nature* **224**, 25 (1969).
[44] P. Pratesi and E. Grava, *Adv. Drug Res.* **2**, 127 (1965).
[45] J. M. George, L. B. Kier and J. R. Hoyland, *Mol. Pharmacol.* **7**, 328 (1971).
[46] L. Villa, V. Ferri, E. Grana and O. Mastelli, *Il Farmaco, Ed. Sci.* **25**, 118 (1970).
[47] L. B. Kier and E. B. Truitt, *J. Pharm. Exptl. Therap.* **174**, 94 (1970).
[48] T. M. Bustard and R. S. Egan, *Tetrahedron* **27**, 4457 (1971).
[49] R. Bergin and D. Carlstrom, *Acta Cryst.* **B24**, 1506 (1968).
[50] L. B. Kier and E. B. Truitt, *Experientia* **26**, 988 (1970).
[51] P. M. Beart, D. R. Curtis and G. A. R. Johnston, *Nature New Biol.* **234**, 80 (1971).
[52] L. B. Kier, *Experientia*, in press.
[53] J. R. Hoyland and L. B. Kier, *J. Med. Chem.* **15**, 84 (1972).
[54] I. Rabinowitz, P. Ramwell and P. Davison, *Nature New Biol.* **223**, 88 (1971).
[55] R. Hoffman and A. Imamura, *Biopolymers* **7**, 207 (1969).
[56] L. B. Kier and J. M. George, *Theoret. Chim. Acta* **14**, 258 (1969).
[57] A. Rossi, C. W. David and R. Schor, *Theoret. Chim. Acta* **14**, 429 (1969).
[58] L. B. Kier and J. M. George in *Molecular Orbital Studies in Chemical Pharmacology*, (L. B. Kier, ed.) Springer-Verlag, New York (1970).
[59] L. B. Kier and J. M. George, *J. Med. Chem.* **15**, 384 (1972).
[60] R. S. Shallenberger and T. E. Acree, *Nature* **216**, 480 (1967).
[61] J. Solms, *J. Agr. Food Chem.* **17**, 686 (1969).
[62] L. B. Kier, *J. Pharm. Sci.* **61**, 1394 (1972).

CONCLUDING REMARKS

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I am greatly honoured by being given this opportunity to summarize the achievements of the Third International Symposium on Medicinal Chemistry. This Meeting has achieved such a uniformly high standard that it is quite unnecessary for me to refer to particular contributions. Therefore I shall give some general impressions.

It was refreshing to find the word *Selectivity* in the title of the very first paper. It is a word which cannot be heard too often in medicinal chemistry meetings, because, no matter how striking the physiological effects of a new drug may be, it can have no medicinal importance unless it is selective.

The present Symposium represents a tremendous change in content from most of the earlier medicinal chemistry meetings. It was the custom for the latter to be almost entirely chemical in nature, with only dark hints that the substances described had biological action. This Symposium, however, has leant the other way by moving chemistry rather into the background. We should be glad of the opportunities, given us during the last three days, to understand how drugs are acting, at the biochemical and biological levels. Yet it may be advantageous for some of the Symposia in this series to keep chemistry a little more in the foreground.

Each of the three topics of the present Symposium was a contribution of great relevance to the present state of our subject. The first day was devoted to discussions of *The biochemistry of microorganisms as a basis for the rational development of anti-infective agents*. On that day we had laid before us much of the most fundamental work now being done on the action of antibiotics. The only significant omission seemed to be the anti-infective agents of entirely synthetic origin, e.g. the nitrofurans and nitro-imidazoles, whose mode of action has never been adequately discussed. The subject of our second day, *Synthetic analogues of the biochemical messengers*, helped us to realize that hormones act only through a long chain of biochemical reactions at the end of which come cyclic-AMP and the prostaglandins. The absence of the latter from the programme is apparently due to the prostaglandin symposium in Vienna next week. Our final day, on *Physicochemical properties and biological action*, reminded us of the many important contributions which the physical chemist can make to the design and perfection of new drugs. The only danger in this approach is that calculations, if not repeatedly cross-checked with experiment, can go far astray. The Baconian revolution of four centuries ago placed Science on an entirely experimental basis, and the progress of Science without constant reference to experiment is, by definition, impossible.

Discussion, throughout the present Symposium, was very good and it grew in intensity as the conference proceeded. There can be no doubt that

this meeting has taken place at a magical moment in the history of our subject, namely at the time when the first agonist-receptor, namely the one for acetylcholine, has been isolated and purified.

The Società Italiana di Scienze Farmaceutiche has earned the thanks of all participants by the splendid organization of the programme and also the social events (formal and informal) during which many valuable personal contacts were made.