PROCEEDINGS of the 5th INTERNATIONAL SYMPOSIUM
on PHARMACEUTICAL SCIENCES (ISOPS-5)

24-27 June, 1997
ANKARA

Editors
Seçkin ÖZDEN
Maksut ÇOŞKUN
Erdem BÜYÜKBİNGÖL
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Special Issue of JOURNAL of FACULTY of PHARMACY ANKARA UNIVERSITY
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ISBN 975-482-363-9

Ankara Üniversitesi Basım ve 1997
PREFACE

Ten years ago we believed that a valuable contribution to the pharmaceutical science area, could be made by bringing together of well-known scientists in their fields of research, within an International Symposium of Pharmaceutical Sciences (ISOPS) to be held in Ankara. This year, we are so happy that the fifth of the Symposium series will be achieved with the contribution of several scientists from all over the World.

In organizing this symposium we decided to try to set the frontier topics, alongside some of the older ones. As you may see from the proceedings, two points quickly emerged that these groupings are not separate but merge or overlap. We feel that these topics will be of interest to research workers and others interested in the discovery of natural and synthetic products and in their further utilization as drugs, pharmacological, clinical and technological tools. We hope that decision makers in industry and in academic areas will benefit from these timely reviews and consider these pharmaceutical science topics as worthwhile endeavours for further research. So that as Organizing Committee, we thank to speakers for so expeditiously providing manuscripts and tolerating our editorial enquiries.

As a product of Faculty of Pharmacy, Ankara University, this book contains proceedings from the 5th International Symposium on Pharmaceutical Sciences which took place in Ankara, Turkey in June 24-27, 1997. The symposium consisted of thirty-two plenary lectures and most of them were adapted as full length papers in this volume. There were about 300 participants whose some of them joined us from all over the World including Asia, Australia, Europe, North and South America and South Africa.

The symposium was conducted under the sponsorship of the Ankara University, The Scientific Research Council of Turkey (TUBITAK) and certain companies from Turkish Pharmaceutical Industry. With this opportunity I would like to express our special thank to Turkish Pharmaceutical Industry for their generous sponsorship in which NOVARTIS Inc. additionally supported to publish the Proceedings book. The success of the symposium also depends on the critical support of the The Scientific and Technical Research Council of Turkey (TUBITAK) in providing funds for expenses for many of the academic speakers and others.

As usual, this book is the result of efforts by many individuals. We benefited enormously during the preparation of this proceedings from many helpful discussions from colleagues at the Faculty of Pharmacy. In particular, we wish to thank to Organizing Committee members for their roles in processing the Symposium and preparation of the material to be included in this book. In addition we would like to thank to other committee members for their contribution to the preparation and evaluation of manuscripts.

Lastly, and perhaps most importantly, we express our sincere appreciation to all participants, whose interest in the field of Pharmaceutical Sciences and active participation assured the success of the symposium and who were gracious enough to write or call with flattering comments.

The year, 1999, will be a very special year for everyone. One step beyond is the beginning of a new era. We all hope to meet you in Ankara for ISOPS6.

Prof. Dr. Seçkin ÖZDEN, Dean
President of Symposium
## CONTENTS

<table>
<thead>
<tr>
<th>TITLE(S) &amp; AUTHOR(S)</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEW TRENDS IN CHIRAL HPLC SEPARATION TECHNIQUES</td>
<td>1</td>
</tr>
<tr>
<td>Hassan Y. Aboul-Encin</td>
<td></td>
</tr>
<tr>
<td>MASS SPECTROMETRY IN CLINICAL AND FORENSIC TOXICOLOGY</td>
<td>17</td>
</tr>
<tr>
<td>Dominique Fompeydie</td>
<td></td>
</tr>
<tr>
<td>SEQUENTIAL INJECTION EXTRACTION WITHOUT PHASE SEPARATION FOR SAMPLE PREPARATION AND DRUG ANALYSIS</td>
<td>32</td>
</tr>
<tr>
<td>Gary D. Christian</td>
<td></td>
</tr>
<tr>
<td>PHYSIOLOGICAL CHEMISTRY OF α, β ANOMERS AND ITS ALDEHYDE FORM OF D-GLUCOSE</td>
<td>43</td>
</tr>
<tr>
<td>Jun Okada and Ichitomo Miwa</td>
<td></td>
</tr>
<tr>
<td>PHARMACEUTICAL EDUCATION AND ACCREDITATION IN THE USA</td>
<td>53</td>
</tr>
<tr>
<td>Daniel A. Nona</td>
<td></td>
</tr>
<tr>
<td>CONTROL OF LIPOGENESIS</td>
<td>69</td>
</tr>
<tr>
<td>David M. Gibson</td>
<td></td>
</tr>
<tr>
<td>ANTIBIOTIC RESISTANCE: A WORLDWIDE PROBLEM</td>
<td>96</td>
</tr>
<tr>
<td>H. Erdal Akalin</td>
<td></td>
</tr>
<tr>
<td>VANADIUM: A POTENTIAL THERAPEUTIC AGENT FOR DIABETES</td>
<td>92</td>
</tr>
<tr>
<td>J.H. McNeill and S. Verma</td>
<td></td>
</tr>
<tr>
<td>MOLECULAR MECHANISMS OF INSULIN RESISTANCE, STRUCTURE AND SYNTHESIS OF A NOVEL INOSITOL-GLYCAN PSEUDO-DISACCHARIDE FROM BEEF LIVER WITH INSULIN-LIKE BIOACTIVITY IN VITRO AND IN VIVO.</td>
<td>109</td>
</tr>
<tr>
<td>STUDIES ON NATURALLY OCCURING SUBSTANCES FOR INHIBITORS OF GLYCOSIDASES</td>
<td>117</td>
</tr>
<tr>
<td>Genjiro Kusano</td>
<td></td>
</tr>
<tr>
<td>MEANINGS OF GENERALLY EXISTING NATURAL COMPOUNDS</td>
<td>129</td>
</tr>
<tr>
<td>Yukio Oghara, Makoto Inoue and Mitsuhiko Nese</td>
<td></td>
</tr>
<tr>
<td>QUALITY CONTROL AND STANDARDIZATION OF PHYTOPHARMACA</td>
<td>132</td>
</tr>
<tr>
<td>Otto Sticher</td>
<td></td>
</tr>
<tr>
<td>COMPLEX TETRA AND PENTACYCLIC COUMARIN DERIVATIVES FROM THE GENUS ERIOSTEMON (RUTACEAE)</td>
<td>134</td>
</tr>
<tr>
<td>Peter G. Waterman, Mohammad A. Rashid and Satyajit D. Sarker</td>
<td></td>
</tr>
<tr>
<td>Title</td>
<td>Author(s)</td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>THE QUANTIFICATION OF STERIC EFFECTS IN QSAR BY THE SEGMENTAL METHOD</td>
<td>Marvin Charton</td>
</tr>
<tr>
<td>ASYMMETRIC SYNTHESIS OF CARBOHYDRATES</td>
<td>John M. Gardiner and Martin Penny</td>
</tr>
<tr>
<td>NEW PERSPECTIVES FOR THE TREATMENT OF HIV INFECTION (AIDS)</td>
<td>Lieve Nuesens and Erik De Clercq</td>
</tr>
<tr>
<td>DESIGN AND SYNTHESIS OF TRICYCLIC NUCLEOSIDES (DIMENSIONAL PROBES)</td>
<td>Leroy B. Townsend, Zhijian Zhu and John C. Drach.</td>
</tr>
<tr>
<td>IN VITRO TESTING PROCEDURES FOR LOCALLY APPLIED/LOCALLY ACTING</td>
<td>P. Loos and H. Möller</td>
</tr>
<tr>
<td>PRODUCTS - SUBSTITUTE FOR BIOAVAILABILITY/BIOEQUIVALENCE STUDIES?</td>
<td></td>
</tr>
<tr>
<td>REGULATORY REQUIREMENTS OF NOVEL DOSAGE FORMS</td>
<td>A.P. Sam</td>
</tr>
<tr>
<td>MODERN TABLET EXCIPIENTS: PRESENT STATUS AND TRENDS</td>
<td>Peter C. Schmidt</td>
</tr>
<tr>
<td>THE ROLE OF THE HEME OXYGENASE SYSTEM IN THE MOLECULAR DYNAMICS OF</td>
<td>James F. Ewing, Tulay Coban and M.D. Maines</td>
</tr>
<tr>
<td>MAMMALIAN GASEOUS MONOXIDE CELL SIGNALING</td>
<td></td>
</tr>
<tr>
<td>THE EMERGENCE OF PHARMACOEPIDEMIOLOGY AS A DISCIPLINE</td>
<td>Jack E. Fincham</td>
</tr>
<tr>
<td>FORMATION OF HETEROCYCLES IN THE MASS SPECTROMETER</td>
<td>Klaus K. Mayer, Herwig Pongratz, Thomas Fürst and Wolfgang Wiegrebe</td>
</tr>
<tr>
<td>TRADITIONAL FOLK MEDICINES &amp; NATURAL RESOURCES FOR ADULT-DISEASE</td>
<td>Toru Okuyama</td>
</tr>
<tr>
<td>PREVENTION</td>
<td></td>
</tr>
<tr>
<td>METABOLISM OF ANANDAMIDE, AN ENDOGENOUS LIGAND FOR CANNABINOID</td>
<td>Natsuo Ueda, Kazuhisa Katayama, Yoko Kurahashi, Mitsuiro Suzuki, Hiroshi Suzuki, Shozo Yamamoto, and Hisao Kato</td>
</tr>
<tr>
<td>RECEPTORS</td>
<td></td>
</tr>
<tr>
<td>BIOSENSORS: NEW TOOLS FOR PHARMACOLOGICAL AND DRUG ANALYSIS</td>
<td>Jean-Michel Kauffmann</td>
</tr>
</tbody>
</table>
NEW TRENDS IN CHIRAL HPLC SEPARATION TECHNIQUES
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ABSTRACT
Optical activity and asymmetry is widespread in nature. Most of the biochemical processes are
stereochemically controlled and it is therefore not surprising that the optical isomers of xenobiotics and
chiral bioactive compounds can behave differently in terms of pharmacodynamics, pharmacokinetics,
and even toxicological activity. However, most synthetic drugs are still administered as a racemic
mixture, mainly due to claimed cost benefits. The explosive development of enantioselective HPLC
methodology over the last decade has rendered the analysis of chiral drugs in bulk, pharmaceutical
dosage forms and in biological fluids an easy task. Enantioselective bioassays of drugs and their optical
purity are becoming important issues both scientifically as required by the drug regulatory authorities.
In order to design direct enantioselective chromatographic systems one needs a chiral selector which is
electronically bonded to a support surface (e.g. silica gel) or a chiral polymer gel. These
stationary phases must have the ability to form physicochemically transient diastereomeric molecular
complexes with the individual analyte enantiomers. The magnitude of the intermolecular interactions of
chiral selector and analyte enantiomers will result in retention. and the spatial orientation of the chiral
substituents in the diastereomeric molecule complex will allow for enantioselectivity. The
intermolecular binding and driving forces forming the “transition” complexes can be coulombic, dipole-
dipole, charge transfer, hydrogen bonding, and or hydrophobic in character.

Based on this concept, over 100 specialized chiral stationary phases (CSPs) have been developed and
are commercially available. An overview of the various types of these CSPs including the recent ones
are presented.

Key Words: chiral stationary phases, stereoisomers, enantiomers, enantioselective assay, chiral HPLC,
chiral recognition mechanism(s).
INTRODUCTION

Chirality arises from an element of asymmetry in a molecule that may be a center, an axis, or a plane. Molecules that are not superimposable on their mirror images are chiral, a class of stereoisomers called enantiomers. Enantiomers have identical physical properties, except for the rotation of optically active or polarized light. Chemical properties are identical as well, except in a chiral environment, for example, in biological systems. Enantiomers are frequently distinguished by biological systems and may possess significantly different pharmacokinetic, pharmacologic, and toxicologic properties. It is for this reason that the development of drugs as pure enantiomers rather than racemic mixtures is an emerging trend in many pharmaceutical companies [1].

A number of different methods are available today for optical resolution purposes [2]. In addition to the classical procedures of making diastereomeric salts of acids or bases, a number of other potentially useful techniques exist, not the least of those relying on chromatographic separation [3]. Furthermore, methods based on resolution by preferential crystallization (also called resolution by entertainment and applicable to racemates crystallizing as conglomerates), recrystallization of cleavable diastereomeric derivatives, and kinetic resolution by enzymes or microorganisms may also be worth considering. An overview of the various separation techniques of drug racemates are shown in Table 1.

Table 1. Potentially Useful Methods for the Optical Resolution of Drug Racemates

<table>
<thead>
<tr>
<th>Method</th>
<th>Possible Applicability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classical resolution by crystallization of</td>
<td>Racemic acids and bases. The technique has also been used after derivatization of the</td>
</tr>
<tr>
<td>diastereomeric salts</td>
<td>target compound, for example resolution of alcohols as monophthalates.</td>
</tr>
<tr>
<td>Separation of covalent derivatives</td>
<td>Reported cases include resolutions of aldehydes and ketones as methylhydrazones, of</td>
</tr>
<tr>
<td>obtained from an optically active reagent</td>
<td>alcohols as various carbonates and carboxylates, of alkenes, sulfoxides, phosphines,</td>
</tr>
<tr>
<td></td>
<td>and other metal coordinating compounds as zerovalent metal complexes, etc. [3].</td>
</tr>
<tr>
<td>Preferential crystallization (resolution by</td>
<td>Racemates crystallizing as conglomerates [4].</td>
</tr>
<tr>
<td>entainment)</td>
<td>Kinetic resolution</td>
</tr>
<tr>
<td></td>
<td>Mainly hydrolyzable substrates for use with esterases, lipases, amidases, etc.</td>
</tr>
<tr>
<td>Chromatographic resolution</td>
<td>In the indirect mode to achieve the separation of diastereomeric derivatives or in the</td>
</tr>
<tr>
<td></td>
<td>direct mode employing a chiral stationary phase</td>
</tr>
</tbody>
</table>
Chromatographic enantioseparations, particularly resolution by high-performance liquid chromatography (HPLC), have advanced considerably in the past decade and become a practically useful method for not only determining their optical purity but also obtaining optical isomers especially in the pharmaceutical industry. Chiral HPLC is well recognized to be useful for the research and development of chiral drugs [5,6]. The preparation of a chiral stationary phase (CSP) capable of effective chiral recognition is the key to this separation technique. Therefore, many CSPs for HPLC have been prepared [3, 7-10] and about 100 of them have been commercialized. Large-scale, preparative HPLC systems have already been put on the market as a process for the isolation and purification of chiral drugs and natural products.

Chiral Stationary Phases (CSPs)

A great deal of progress have been made with the development of wide variety of CSPs which are commercially available [11-14]. These CSPs operate through the formation of transient diastereoisomers via interactions between both enantiomers and the chiral selector as shown in Figure 1. In case of CSP, the chiral selector is immobilised, mostly on a silica support. The chiral recognition mechanisms operating on these phases are the result of the formation of temporary diastereomeric complexes between the enantiomeric solute molecules and the immobilised selector. The total chiral recognition mechanism between the solute and the chiral selector can be split up into two parts, keeping in mind that they are actually inter-dependent:

- the formation of the solute/CSP-complexes
- the expression of stereochemical differences during and after formation of these transient diastereomeric complexes.

The formation of the complex is generally accepted to proceed through a three-points interaction mechanism, which in a simplified way, is displayed in Figure 1. The possible type of interactions between the selector and selectand are shown in Table 2.
Table 2. Possible types of interactions required for chiral recognition

<table>
<thead>
<tr>
<th>Selector (CSP)</th>
<th>Selectand (Analytes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>electron donor</td>
<td>electron acceptor</td>
</tr>
<tr>
<td>π-donor</td>
<td>π-acceptor</td>
</tr>
<tr>
<td>Hydrogen donor</td>
<td>Hydrogen acceptor</td>
</tr>
<tr>
<td>dipole</td>
<td>dipole</td>
</tr>
<tr>
<td>cation</td>
<td>anion</td>
</tr>
<tr>
<td>attraction</td>
<td>repulsion</td>
</tr>
</tbody>
</table>

Figure 1. Three point interaction model of chiral recognition and resolution
These CSPs has been classified to five types as shown in Table 3 [15].

Table 3. Classification of Chiral Stationary Phases.

<table>
<thead>
<tr>
<th>Type</th>
<th>Description</th>
<th>Chemistry</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Brush (Pirkle type) $\pi$-acceptor type</td>
<td>Various chiral selectors ionic or covalent bonding</td>
<td>Attractive interactions hydrogen bonding Charge transfer (n-\pi interaction) Dipole stacking and steric effects</td>
</tr>
<tr>
<td>II</td>
<td>Helical Polymers</td>
<td>Cellulose derivatives</td>
<td>Attractive interactions Inclusion complexes</td>
</tr>
<tr>
<td>III</td>
<td>Cavity</td>
<td>Cyclodextrin Polymethacrylates Polyacrylamides</td>
<td>Inclusions complexes</td>
</tr>
<tr>
<td>IV</td>
<td>Protein</td>
<td>$\alpha$-acid glycoprotein Human serum albumin Ovomucoid $\alpha$-chymotrypsin Cellobiohydrolase</td>
<td>Hydrophobic interactions Polar interactions</td>
</tr>
<tr>
<td>V</td>
<td>Ligand Exchange</td>
<td>Amino acid-metal complex</td>
<td>Diastereometric metal complex</td>
</tr>
</tbody>
</table>

I. Pirkle Type Phases

Pirkle and co-workers [13, 16] pioneered the development of a variety of CSPs consisting amino acid derivatives, typically N-(3,5 dinitrobenzoyl) phenylglycine (DNBPG), immobilized on silica either ionic or covalent attachment Fig. 2. These phases are classified into $\pi$-acceptor or $\pi$-doner phases [17]. Many of these columns are commercially available and widely used for analytical and preparative scale separation of multi kilo quantities.

These phases are stable at high pressures and exhibit good chromatographic performances. Usually Pirkle type phases are used under normal phase conditions but it has also been demonstrated that chiral resolutions of drugs could be achieved in the reversed phase mode [18-20].
II. Helical Polymers Phases

These include a wide range of cellulose and amylose based phases Fig. 3. The high potential of these CSPs is clearly demonstrated by good separation of a broad range of racemates by using these polymer adsorbed on macroporous silica [21-24].

The most widely used derivative for separations on a preparative scale is cellulose triacetate (CTA-1) which was introduced by Hesse and Hagel in 1973 [25]. CTA-1 has been extensively used to resolve a broad range of structurally different racemates because of its high loading capacity, low preparation costs as compared to other phases of this class of CSPs such as the 3,5-dimethylphenyl carbamate cellulose or amylose derivatives which are expensive. The cellulose and amylose based CSPs are used under both normal and reversed phase conditions.
III. Cavity Phases

A. Cyclodextrins

Cyclodextrins are cyclic oligosaccharides (Figure 4) having the property of forming stable inclusion complexes in their highly hydrophobic cavity (chiral host) with a wide variety of molecules [26]. Owing to the inherent chirality of their building units, namely the α-1, 4-linked glucose moiety, these cyclic oligosaccharides are chiral and the formation of diastereomeric complexes with the two enantiomers of racemic compounds can be very stereoselective.

The size of the cavity, which differs for α-, β-, and γ-cyclodextrins and the substituent on cyclodextrin, plays a decisive role in determining the ability of these oligosaccharides to complex a defined molecule. Cyclodextrins CSPs were prepared by immobilizing CD in polymeric structures [27, 28], or in silica gel [29, 30], the latter CSPs showing good performance on an analytical scale. Preparative applications using cyclodextrins as chiral hosts were reported on polymers obtained by cross-linking of cyclodextrins with ethylene glycol.

**Figure 3.** Structure and geometry of the cellulose and amylose CSP’s
bis(epoxypropyl) [28] and on the silica-modified materials Cyclodond l [31] and Chiral Dex [32].

Although reversed-phase conditions are usually applied with the cyclodextrins-based CSPs, analytical applications in the normal mode have also been reported [33]. Although cyclodextrin derivatives have also been introduced and turned out to exhibit a better chiral recognition for some classes of racemates [34], limited preparative separation on these CSPs has been reported up to now.

![Structures of cyclodextrins]

Figure 4. Structures of cyclodextrins
B. Polymethacrylamide

Polymethacrylamide constituted another class of the cavity phases. The bulky triphenylmethyl groups are assumed to direct the growing polymer chain to take a helical conformation [7, 35]. Fig. 5. Blaschke and co-workers [36] demonstrated the usefulness of this phase for preparative applications.

\[ \text{Fig. 5. Structure of polymethacrylate CSP's} \]

IV. Protein Phases

A number of CSPs have been developed by immobilizing proteins or enzymes such as bovine or human serum albumin [37, 38], α-acid glycoprotein [39], α-chymotrypsin [40], cellulase [41] and, ovomucoid [42] on silica gel. These CSPs usually operate under reversed-phase conditions (phosphate buffers with addition of organic modifiers). As would be expected from a protein, the separation depends very much on parameters such as pH, ionic strength, temperature, the concentration or nature of the modifier. Although these phases separate a wide range of racemic drugs yet their low loading capacity and high costs render them, not
very attractive for preparative scale chromatography. However, a few semipreparative applications had been reported on the α-1-acid glycoprotein [43, 44].

V. Chiral Ligand Exchange Phases

In chiral ligand exchange chromatography, introduced by Davankov and co-workers [45, 46], an optically active ligand-usually an amino acid is covalently bound to a solid support. Chloromethylated polystyrene was used originally; subsequently, Gubitz and co-workers [47] developed silica-bound CSPs (e.g., L-proline attached to silica by a 3-glycidylpropyl spacer). After loading the adsorbent with Cu$^{2+}$ or Ni$^{2+}$ ions, the racemate is chromatographed. Each amino acid molecule displaces one of the polymer-bound proline ligands to form a mixed complex (Figure 6).

![Figure 6. Chiral ligand exchange chromatography](image-url)
Vancomycin and teicoplanin are macrocyclic antibiotics that have been covalently bonded to a silica gel support and serve as chiral stationary phases (CSPs) for high-performance liquid chromatography (HPLC). Their commercial names are Chirobiotic V and T, respectively. The number of antibiotic macrocycles available for such a purpose is high, giving many possibilities for developing several CSPs.

Chirobiotic V and T have been shown to be effective chiral selectors for the enantiomeric separation of many racemic drugs [48, 49]. These phases are resilient and are compatible with the usual HPLC solvents in practice, for reversed- and normal-phase applications. The chemical structures of these antibiotics (Figs. 7 and 8 respectively) contain numerous functionalities and several active pockets which contribute to the chiral recognition mechanism(s) responsible for effective enantiomeric resolution. For example, Chirobiotic V selectivity characteristics include but are not limited to: 1) a complex chiral environment 2) a carbohydrate binding site 3) inclusion complexation 4) a peptide binding site 5) chiral π-π interactions 6) chiral hydrogen binding sites and 7) ionizable groups [50]. Also, these chiral stationary phases are not limited in application as the protein or cellulose phases are.

![General physical data: Molecular weight - 1449
pKₐ - 4.9, 7.2, 8.6, 9.0, 10.4, 11.7
Isoelectric point - 7.2
Chiral centers - 18
Inclusion cavities A, B, C

Figure 7. Proposed structure of the macrocyclic glycopeptide vancomycin](image-url)
General physical data:
Molecular Weight - 1885
Chiral Centers - 20
Sugar Moieties - 3
Fused Rings - 4 (A,B,C,D)
R - CH3-decanoic acid

Figure 8. Proposed structure of the macrocyclic glycopeptide teicoplanin

Chirobiotic V and T are complimentary phases in that if a racemic compound is not enantiomerically resolved on one phase, the other phase may show an increase in selectivity for the same racemic compound under the same mobile phase conditions. This feature further enhances the possibility of studying racemic compounds under the same chromatographic conditions.

Macrocyclic antibiotics offer viable alternative CSPs, considering the hundreds of macrocycles that are available for CSP production and the numerous chiral recognition mechanism they possess based on their multifunctional and complex chiral chemical structures.
CONCLUSION

The advantages of applying a chromatographic process for the direct resolution of drug racemates for the purpose of analysis in bulk powder form, pharmaceutical formulations or biological fluids or on industrial scale over the classical methods using these chiral stationary phases are:

1. When the chiral stationary phase used is completely immobilized, no loss of valuable optically active materials can occur.

2. If the chromatographic peaks are fully resolved, both enantiomers can be quantitatively obtained, 100% pure.

3. Recovery of the eluted material can be easily automated.

ACKNOWLEDGEMENT: The author (H.Y.A.E.) wish to thank the Administration of King Faisal Specialist Hospital and Research Centre for their continuous support to the Bioanalytical and Drug Development Research Program.
REFERENCES

Chichester/New York - Horwood/Wiley.
Weinheim, VCH Publisher Chapter 11, 1994.
VCH Publisher.
(1983).
Ellel, Otsuka (Editors). Asymmetric Reactions and Processes in Chemistry, ACS
ABSTRACT
Since devices easy to use are available, GC-MS can be applied to screen drugs and toxic. It permits the unequivocal identification of drugs. Protocol for its use for illicit products is classical but in biological material, matrix and drug metabolism require special protocol:
* hydrolysis (acid, basic or enzymatic) to dissociate conjugates
* separation of the drug and its metabolites by extraction (liquid-liquid or liquid-solid)
* derivatization to convert polar groups into non polar derivative to improve chromatography and increase sensitivity.

The sample is then submitted to chromatographic separation and mass analysis.
The answer can be done by comparison of retention time and mass spectrum stored in library with reference standard.

Quantitative analysis using an internal standard can be made by SIM acquisition.
We present some results of analysis done in our laboratory.

KEYWORDS : Mass spectrometry, drugs, toxic, drugs of abuse.
INDRODUCTION

With the development of cheap and easily manipulated GC-MS instruments, the use of this technique to screen drugs has constantly been increasing recently. In fact, it proved to be extremely efficient thanks to its specificity and its sensitivity and in some cases quite essential, however its use in clinical and forensic toxicology involves some technical problems of adaptation which are not actually the same in these two fields.

The first problem is the time needed for analysis by mass spectrometry. In forensic toxicology, when it concerns a dead patient, long lasting analysis is not anymore the limiting factor. On the other hand an unquestionable proof of the identification of the incriminated product is required and mass spectrometry is needed with microcomputers linked to the devices which allows the editing of written documents. Usually, emergency toxicology requires a quick answer and mass spectrometry is most of the time used subsequently. Indeed, the physician is directed by the clinical symptoms and at first he needs the confirmation of the class of the suspected toxic agent as the first symptomatic treatments are generally the same for one group of poisons. We can get this first answer by immunochemistry for example. Later, mass spectrometry allows the confirmation the exact nature of the product which is very important for example when there is suspicion of poisoning or of drug abuse, some products may give false positive reactions by immunochemistry.

Products analysed in the laboratory

Products submitted to analysis in the laboratory are of varied nature and the treatment prior to analysis differs with each case. We may have to deal with tablets, capsules, powders, dried or not syringes, pipes, liquids and also for example a cake. In all cases, the researched substances will be pure or approximately so and in their original state. Concerning tablets after grinding or powders, we dissolve about 3 mg in 3 ml of methanol. The dry syringes or the pipes, are rinsed with the same solvent. For the non aqueous liquids, we dilute them in the methanol. If they are aqueous, an extraction is needed. An exception: alkyl nitrites
(constituents of "poppers") which are easily recognisable by their characteristic smell and for which we make a partial injection without previous dilution because their chromatographic behaviour is too near that of usual solvents. The analysis is done by injection of the solution after dilution in ethanol or if necessary after concentration or derivatisation.

The second group of samples constitutes of samples of biologic origin: stomach wash content, blood, urine, hair, saliva and in forensic toxicology viscera. First, it is necessary to pay a particular attention to the conservation of the samples, the results depend on the efficiency of the analysis. The taking will be stored in a refrigerator or a freezer.

In the laboratory, the most usual samples are urine. Indeed, the concentration of drug is important there and the taking of urine is not an invasive gesture; it is an asset in particular when it comes to the research of drug of abuse.

**Treatment of biological products**

Prior to the analysis of the compounds, we must consider the transformations that the molecule undergoes in the organism in the process of metabolism. This consists of a set of biotransformations in which the ultimate objective is to obtain soluble molecules in order to permit their elimination in bile or urine. It is completed within two phases, most often the first one consists in an oxidation conducting to some hydroxylated derivatives or to some dealkylated derivatives or in an hydrolyse of the esters or amides. The second phase transforms the previous products in hydrosoluble compounds. The main reactions are conjugations: glucuronococonjugation, sulfoconjugation for the hydroxylated groups or acetylation for the amino groups. According to the origin of sample, the nature of recovered products will be different: in a stomach wash content, one will recover the initial product (except for the products degraded in acidic medium); in blood and hair, one will recover the product and some metabolites often bound to proteins; in urine mainly conjugated metabolites will be found.
Besides, the proportions of the different metabolites will change according to the metabolism kinetics (generally expressed in half-life time) and the time interval between the ingestion of the product and the taking of the biologic sample. If the elimination of drug is very fast, like in the case of LSD for example, you cannot expect to detect it in the sample of urine taken a few hours after the intake.

Therefore these biological samples are very complex. They contain many compounds at concentration greater than that of the analysed product. Consequently, it is necessary to carry out a stage of isolation which will be the most selective as possible. Most often, it consists in an organic solvent extraction. We saw that in urine drugs were in hydrosoluble forms. Therefore before proceeding the extraction, it is necessary to break this conjugation. The hydrolysis could be chemical or enzymatic. Acidic hydrolysis is convenient for most of samples. It is achieved in the presence of concentrated acid (hydrochloric) flowing back for 15 to 30 minutes. The disadvantage is that all the hydrolysable links will also undergo the hydrolysis. For example, during the metabolism of heroin, 6 monoacetylmorphine is formed which confirms the intake of that drug. If we want to screen this metabolite, a chemical hydrolysis which will transform it in morphine, a metabolite of many opiates like codeine, cannot be used. Enzymatic hydrolysis is a lot softer and more specific [1]. The samples are treated with glucuronidase and sulfatase during at least 12 hours at 37°C or 3 hours at 50°C. Only the corresponding esters are hydrolysed. Some studies compared the reactivity of enzymes from different origin in relation to the nature of the ester linkage. In the case of the Δ9 carboxy THC -main metabolite of tetrahydrocannabinol-, a chemical hydrolysis with potassium hydroxide 1M can be made.

As or hair, they are first submitted to a washing with aqueous buffered solutions and water-isopropanol mixtures in order to avoid external contamination. Then, we let a proteinase operate at 37°C for one night in order to free the substance of any possible protein links.
Isolation

Then, we can proceed to an extraction. If we know what we are searching, which is the most frequent case for biological samples in the laboratory, mass spectrometry happens to confirm the result obtained by another method like the immunochemical analysis, the extraction will be selective. One relies on the ionisation of the product according to its pKa and pH, the molecules being generally soluble in organic medium, whereas the ionised forms are not. If nothing is known, extractions with alkaline and acidic pH with at least two different solvents must be undertaken.

The most used solvents are chloroform, dichloromethane (less toxic in the long run), ethyl acetate, diethyl ether. In some cases we can use some more specific solvents: butyl/chloride for LSD, mixture of chloroform/isopropanol for opiates.

We can make liquid/liquid extractions provided we manage to avoid the formation of emulsion. Varying the pH, different stages of washing can be achieved. For example, in order to isolate a product with basic character (which is the case of most of the compounds which interest us), it is possible to achieve a first extraction with an acidic pH. Our product remains in aqueous phase, organic phase is eliminated and aqueous phase is made alkaline in order to extract the researched product in an organic solvent; this technique particularly permits the elimination of some molecules like fats acids ...

For acidic drugs like paracetamol or some barbiturates, an extraction with an acidic pH will be performed. Anyway, the extract remains rather rich even in the absence of drugs or of medicines and this all the more so when a solvent of little specificity (diethylether for example) is used.

Liquid/solid extractions are now widely used [2,3]. This involves chromatography achieved in small columns which are going to keep or to selectively elute the products according to their nature and the pH used. The usual process for screening basic products in the laboratory is based on this system. We use a support named Extrelut* and urine buffered at pH 9.2 is added.
After 20 min, the elution is accomplished with chloroform. There again, numerous substances are extracted because retention is not specific. Columns filled with more specific phases can be used [4]. For example we use Narc2* columns (a bed mixed with cation exchanger) for the extraction of cocaine and of its metabolites. We can notice that the extracts are then much purer. The advantage is that these systems require less solvent but their cost is not negligible. Besides, it is necessary to work under a light vacuum and the columns will be conditioned in an appropriate way before the sample is added, moreover some systems exist that allow work on several columns at the same time.

**Figure 1**: GC-MS analysis of an urine with cocaine and metabolites extracted on a Narc 2* column

Then, the solvent is evaporated under nitrogen with moderate heating (37°) excepted in case of more volatile products like amphetamines.

In order to control the good output of the different steps, it will be of interest to add an internal standard at the very beginning of the process, which will also be used for the mass chromatography step. The ideal is to use a corresponding deuterated compound or otherwise, to take a product of close structure but giving a well-separated peak in chromatography, yet we must be careful not to choose a potential metabolite.
The residue of evaporation is dissolved with methanol before being injected directly. However, in the case of lesser volatile or very polar products, it is necessary to improve the chromatographic analysis performing a derivatisation which replaces mobile hydrogens with various groups (silyl, alkyl etc.). It will also be interesting to derivatise in order to improve the identification of molecules having weak mass like amphetamines. It is necessary then to dissolve the residue of evaporation in an aprotic solvent and let the derivatisation reagent (MSTFA, BSTFA, acetic anhydride...) react in a closed bottle at hot temperature. To avoid the contamination of the detection system by the reagent, it must be evaporated before injection; it is necessary to do so just before the injection, the derivatives being generally unstable without presence of an excess of reagent and then we mix the residue with an aprotic solvent. However, in the case of very unstable compounds, like the silylated derivative of LSD, the reagent cannot be evaporated before injection.

![Mass spectra of metamphetamine derivatised (with propylchloroformate) and no.](image)

**Figure 2**: Mass spectra of metamphetamine derivatised (with propylchloroformate) and no.

We must bear in mind that drugs are often modified by metabolism process before being eliminated. This is going to have several aftermath:
- it is necessary to take into account the half-life time of the product, some of them have very short half lives and if the sample is taken too late, we won't be able to recover them (L.S.D...).
- most of the time the metabolites will be recovered by analysis. If the metabolite is common to several active principles (case of benzodiazepines), this can be a drawback.

On the other hand the identification of the metabolites can undoubtedly confirm the intake of drugs (6-monoacetylmorphine for heroine...), or the concomitant take of several products (cocaethylene for cocaine and alcohol). For routine screening, we can perform a basic extraction without previous hydrolysis nor derivatisation, many drugs being identified thus.

**Separation methods**

In spite of stages of extraction, we saw that the samples submitted to analysis by mass spectrometry remain complex. Therefore it is necessary to separate them before arrival in the mass spectrometer source.

To this end, most often gas chromatography commonly employed for the separation of organic substances is used. This allows an increase in the selectivity of the technique and adds chromatographic information -like retention time or the more reproducible value; relative retention with regard to a standard- to the identification of the product.

The use of capillary column solved the former difficulty of coupling the chromatograph with the mass spectrometer due to difference of pressure between the chromatograph and the inlet system : the chromatograph functions with a pressure superior to 1 atmosphere whereas the inlet system of the spectrometer is under vacuum of about 10⁻⁷ torr. Those column work with a flow rate of carrier gas from 1 to 2 ml/ mn and they don’t need a separator, therefore transfer all the effluent in the ionisation source. Nonetheless, we must work with 25 meter long columns in order to avoid some loss of resolution due to the fact that the extremity of the column will be at a lower pressure than an atmosphere. Between the exit of the chromatograph...
and the ionisation source, the column is included in a heated transfer line in order to avoid the impairment the chromatographic separation.

The most commonly used injection methods are either "split splitless" or "Ross injector" (injector with moving needle). At all events, the solution is introduced through a septum with microsyringe; in the injector "split splitless", injected solution is vaporised in the injection port. In "split" mode, only a chosen percentage of this vapour is introduced into the column, which could be an inconvenience in the case of traces analysis, whereas with the device "splitless" all the sample is introduced. This system can be automated. In the case of the Ross injector, the solution is put on the extremity of the glass needle; the carrier gas permits the evaporation of the solvent before putting down the needle in the injector port. This allows to work with solutions of low concentrations, to avoid the solvent injection and to increase the length of life of the columns.

The choice of stationary phase depends on the analysis to perform; usually, polar columns are used for polar compounds and vice versa. However, the shift of column induces an immobilisation of the instrument. In routine analysis we use a column that generally enables us to solve most cases in a satisfactory way, knowing that it may not be the best for a particular case. We use a column of moderate polarity made of silicone bonded with dimethyl (95%) and diphenyl (5%) groups. We must necessary avoid working with extreme conditions of temperature which entails the risk of a distillation of the stationary phase damaging the column and the source.

Most often, the chromatographic method uses monitored temperature programmes in order to have a correct resolution for every compounds, even those with little volatility and within a moderate time of analysis. The program that we apply for routine screening is accomplished in 20 minutes. It could be modified according to the characteristic of the products to analyse (in the case of amphetamines which are very volatile, we begin the programme at a rather low temperature so that the exit is not too fast and vice versa).
Coupling the mass spectrometry and the gas chromatography knows limits due to the latter (low volatile or very polar products...). The high-performance liquid chromatography allowed to palliate these inconveniences, which explains its boom [4]. The problems of its coupling with mass spectrometry, more particularly the elimination of the mobile phase vapour, are now well mastered and this technique begins to be used in toxicological analyses, though the cost restricts its use.

Mass analysis

Most frequently used mass spectrometers are equipped with a quadrupole or with an ion trap detector. Most often ionisation is made in electronic impact mode at 70 ev. This produces many fragments which prove very useful for the identification of the products. We could use chemical ionisation, in particular for finding out the mass of the molecular ion of one compound (barbiturates, benzodiazepines...).

The acquisition can be done in total ion current (TIC) or in single ion monitoring (SIM) mode. In the first case, all the masses are taken into account and the resulting acquisition will show the aspect of a chromatogram and will reveal all the compounds. In the second case, only the products giving fragments having the chosen mass will be detected. Therefore the acquisition will show less peaks and besides, there will be a substantial increase in sensitivity interesting particularly interesting in the case of a quantitative analysis.

The instruments are equipped with data processing program which permits the extraction of ions of chosen mass within a spectrum in total ion current. They also possess spectra libraries. Mass spectra stored in these libraries are generally acquired in electronic impact mode. The libraries contain various numbers of molecules and the research can be more or less time consuming and they can also specialised.
In toxicology, we could mention the Pfleger-Maurer-Weber library [6] which contains most of the substances which interest us and their metabolites. It is also advantageous to the analyst to build up an individual collection of spectra on his own device containing those compounds most likely to be encountered. When we are going to submit a spectrum to the comparison with the spectra stored in the library, a statistical comparison and an indication of the correlation will be performed. One of our instruments displays three values: the "fit" which measures the degree in whom the spectrum of the unknown is inclusive in the library spectrum; the "rfit" which measures the degree with which the library spectrum is inclusive in the unknown spectrum; the "purity" which measures the correlation between the spectrum of the unknown and the one of the library spectrum. 10 products whose spectra are the closest of the spectrum of the unknown are then proposed and their spectra can be visualised. If there is a good score in "fit" but a poor "purity", it may indicate that the unknown spectrum is a mixture which contains the spectrum proposed by the library research.

Figure 3: Mass spectrum of silylated Δ9 THC acid and comparison with spectrum in library
If we search for a given product when the acquisition takes place in total ions, it is necessary to call the masses of some characteristic ions. To confirm the existence of the product, at its own retention time, these masses must be present. The retention time observed will be the same as the one expected for the product accepting a variation at most equal to 2%. It will also be confirmed by comparison with the standard spectrum stored in the library. If we do not know what to search, we will first analyse the peaks observed and compare them with answers from the chosen library. To identify a product, in addition to a statistically correct result, we must get a retention time similar to the one of the proposed product. Screening an unknown product, some authors suggested computerised systematic research for the different classes of products with a selective research of the characteristic mass and then a confirmation by analysis of mass spectrum [7, 8].

Figure 4: Characteristic masses selected from TIC for searching Δ9 THC COOH
Quantitative measurements, most often in mode SIM, are made with an internal standard. This internal standard could be either universal or a corresponding isotope of the drug. It is usually added at first step of the analysis in order to take into account all the parameters which could influence (efficiency of extraction or of derivatisation for example). In order to verify the selectivity of the analysis, one could quantify on several characteristic ions masses (case of the coeluting products).

There again the attitude will vary with demands. In some case, it is not always necessary to have a rigorous quantitation, in case of screening for drugs of abuse it will be necessary to fix a limit below which the result will be ascertained as negative.

**Analytical process**

It will definitely depend on the requests.

The first group of applications concerns screening for drugs of abuse in the setting up of a substitutive programme with methadone or of work place medicine. In this case, the samples are first submitted to the immunochemistry which allows detection of some classes of products (opiates, benzodiazepines, amphetamines...) If the result is positive, this will be confirmed by mass spectrometry which will also enable us to identify the molecule.

For products that we don't search by immunochemistry (palfium, pethidine, LSD...) and when no indication is known, the sample is directly examined by mass spectrometry. We proceed in the same way when there are some doubts concerning a sample, because the addition of some substances to the urine could modify the answer by immunochemistry but not the analysis by mass spectrometry. If we want to reveal a certain product, one can screen for the characteristic ion fragments. However, we usually work in total ion current. This permits the confirmation of the molecule both by observed retention time and by comparison of the mass spectrum with the corresponding spectrum stored in library. On the other hand, we can also bring to the fore other usual products like caffeine which can prove that extraction has been
well done or some other unexpected drugs. For example, this allows us to show that the analysed urine of a patient taking methadone was not in fact his own, the sample containing neither the methadone which he took in the hospital, nor its metabolite.

When these difficulties have been understood, GC-MS is technique of choice for the identification of those compounds. The selectivity of the method can indeed be optimised at different stages: treatment of the sample (selective extractions...), derivatisation, chromatography (change of stationary phase, of temperature programmes), ionisation method.

With some concrete examples one will see that most of the poisons and drugs can be detected by this method, provided by the respect of appropriate conditions.

**Figure 5**: example of GC-MS analysis of an urine

The use of new ways of separation like supercritical chromatography or capillary electrophoresis and the vulgarisation of new technologies like mass-mass analysis allow us to predict new developments in this field.
REFERENCES


SEQUENTIAL INJECTION EXTRACTION WITHOUT PHASE SEPARATION FOR SAMPLE PREPARATION AND DRUG ANALYSIS

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ABSTRACT

Solvent extraction is a powerful technique for sample pretreatment for drug analysis. Analytes may be separated by classes of compounds to improve selectivity, separated from the sample matrix to eliminate matrix effects, or preconcentrated to improve sensitivity. Conventional solvent extraction is time consuming and labor intensive, and generates large amounts of solvent waste. We have combined the technique of sequential injection analysis (SIA) with a thin-film extraction method to perform automated sequential injection extraction (SIE). SIA is an intermittent flow technique in which microliter volumes sample zone and organic solvent zone are sequentially injected next to one another. The organic solvent plug deposits as a thin film on a Teflon tube as it is pushed by the aqueous sample plug, and the sample pushes through the solvent, causing the analyte to extract into the thin film. The flow is reversed and a second extraction step occurs. The process may be repeated with a fresh injected aqueous buffer zone to back extract the analyte. The analyte, in either the organic or aqueous phase, may be transported to a flow cell for direct measurement, or it may be collected for analysis by, e.g., chromatography. The SIE technique has been demonstrated for the extraction and back extraction of barbiturates (acid/neutral compounds) or serotonin reuptake inhibitors (basic compounds), as classes of compounds, for analysis by HPLC. SIE is demonstrated for direct photometric measurement of the extracted analyte, for the determination of molybdenum, vanadium (IV) and (V), and chromium (III) and (VI).

Keywords: micro solvent extraction, sequential injection, thin-film extraction, automated, sample preparation, barbiturates, serotonin reuptake inhibitors
INTRODUCTION

Solvent extraction is a widely used technique for preparing samples for drug analysis. It can be used to separate classes of compounds and removing analytes from the sample matrix, and for preconcentrating them. Conventional solvent extraction involves the use of large quantities of organic solvents, which must be disposed of, and labor intensive manual manipulations.

Flow injection analysis [1] is an efficient solution handling technique that allows automated manipulation of small quantities of solutions, and rapid measurements. Karlberg and Thelander [2] and Bergamin et al. [3] introduced the technique of flow injection extraction (FIE), which allows extraction to be performed using small volumes of solvents. In this technique, aqueous and water immiscible nonaqueous streams merge and segment, and flow through an extraction coil. The sample is injected into the aqueous stream, and extracts into the segmented organic solvent. Following extraction, the organic solvent is separated via a phase separator, either gravity based or membrane based, and is directed to a flow cell for detection and measurement of the analyte. Or, it may be collected for, e.g., gas chromatography analysis. For additional preconcentration, a closed loop continuous extraction system may be employed [4, 5].

Flow injection analysis is a continuous flow technique, and so considerable amounts of waste are still generated. And use of multiple channels can result in complex manifolds, and changing chemical systems can involve extensive physical change of the manifold. The technique of sequential injection analysis (SIA) is a single line system that utilizes intermittent flow, that is, flow only occurs when a sample is being injected and measured [6]. It employs a multiport selection valve in which a common port can be switched to any selected port for aspirating a sample, reagent or solvent, or to a detector port for propelling aspirated solutions to the detector. Representative manifold designs are illustrated below for solvent extraction (see, e.g., Figures-1 and -2). SIA has the advantage of being able to select or alter the chemistry by simple computer control of which ports to access, without physical manipulation of the manifold. Since flow only occurs during the measurement cycle, chemical waste production is reduced. We have applied this technique for solvent extraction and sample preparation, and examples are given below.
In conventional FIE, the two phases may interact differently with the tubing material in the extraction coil. In nonpolar Teflon tubing, the organic segments wet the walls and surround the aqueous segments. Photographic measurements have shown that the portion of the organic segment in the film layer increases with increased flow rate and with increased viscosity to interfacial tension ratio of the organic solvent [7]. Lucy and coworkers [8] noted differences in solutes partitioning into the faster moving aqueous phase and those that partition into the film-forming organic phase when running samples through a long extraction coil. Lucy's laboratory has exploited the temporal as well as spatial resolution of analytes due to the organic film present in FIE [8, 9, 10]. Unextracted compounds present in a faster moving aqueous phase are separated in time from those extracted into the film-forming organic phase. Detection of the time-resolved compounds was done either in a homogenate produced by mixing the aqueous and organic segments with methanol [8], or in the aqueous phase after back extraction [10]. This is an important improvement in FIE that makes unnecessary the physical separation of the two phases and the use of phase separators, which has been a weak component due to fouling and carryover [11].

We have combined sequential injection analysis with the film extraction system of Lucy to perform automated solvent extraction and back extraction for sample preparation and measurement. These may be applied to drug analyses. Example model systems are described below to illustrate the principles and capabilities of the system.

Sequential Injection Extraction for Sample Preparation [12]

The sequential injection extraction (SIE) system has been demonstrated for automated sample preparation of a multicomponent sample, for liquid chromatography measurement. The SIE utilizes the wetting film of organic solvents on Teflon tubing and the resulting differential migration of aqueous and organic phases as described above. A simplified, robust manifold design brings sequentially loaded organic solvent and aqueous sample into contact and resolves them, without the conventional components of phase segmentor and separator. Figure-1 illustrates the manifold design. Reagents and solvents are nested around the multiport valve. The extraction coil is positioned between the reversible pump and the common port. The operation of the system is illustrated by observing methylene blue in an aqueous phase. A 45 ml volume of organic phase (OR) is sandwiched between two 45 ml zones of aqueous methylene blue at pH 12 (Figure-2a). The methylene blue does not extract at this pH. Figure-
2b illustrates that after the zones have traveled a distance, $L$, the organic solvent has all been effectively deposited as a film on the wall. At this point of inversion, the front of AQ2 has traveled through the organic solvent and reached the tail of AQ1.

**SIE Manifold**

![SIE Manifold Diagram](image)

**Figure-1**
Zone Inversion Length, L

a. Zones loaded in the extractor coil

![Diagram of extraction setup](image)

b. Zones at Length, L, where OR has been depleted as a film

Figure-2

The experimental protocol for performing solvent extraction is illustrated in Figure-3. First, a wash solvent to be used at the end of the run is loaded into the coil. An air segment is introduced to separate this from the extracting solvent, OR, which coats the wall as the aqueous phase pushes it down the tube, until it pushes through OR. Air is then pumped in to propel the aqueous sample through the organic solvent, and all of the solvent is deposited on the wall. By then changing the pump direction, the sample is pushed back through OR again by the air segment, the analyte extraction is completed, and the aqueous phase is pushed to waste, leaving the separated organic phase as a film, now containing the analyte. Back extraction into a new appropriately buffered aqueous phase is accomplished by repeating the sequence with the new aqueous phase. The aqueous phase is collected for injection into a liquid chromatograph. At the end of the cycle, the wash solvent has washed the extracting solvent off the tubing, and the system is ready for the next extraction.
The zone inversion length depends on the viscosity of the organic solvent and on the flow velocity [12]. The length increases with decreased flow and decreased viscosity. Low viscosity solvents do not have sufficient difference in flow velocity from that of water, which makes the SIE less efficient, as it requires longer extraction coils and more time. On the other hand, highly viscous solvents are difficult to wash out. Solvent mixtures of intermediate viscosities, near 1 cP, provide sufficient difference in flow velocity from water without adhering too strongly to the tubing. Also, air as propellant lengthens the inversion zone compared with aqueous propellant.
The SIE system was demonstrated for sample preparation, for the determination of barbiturates (acid/neutral compounds) and serotonin reuptake inhibitors (SRIs, basic compounds) in urine, using the manifold shown in Figure-1. In the procedure, 500 ml of urine is buffered at pH 9 with 100 ml of buffer. This is extracted with 50 ml of 4:1 butylchloride:octanol. Back extraction is performed with 100 ml of 0.45 M NaOH for barbiturates or 100 ml of 0.18 M H₃PO₄ for SRIs. The sample and solvent are loaded into the extraction coil at a velocity of 3.3 cm/sec, then air propels the zones 220 cm into the coil, and zone inversion occurs. The analytes are back extracted and collected for analysis by HPLC. The organic film is washed off with 400 ml acetonitrile.

Recoveries using SIE were generally greater than with conventional solvent extraction, with comparable repeatability (ca. 1-7%).

SI Wetting Film Extraction Photometric Determination of Molybdenum [13]

SIE can be used for the direct photometric determination of analytes. The manifold shown in Figure-4 was used to determine molybdenum (VI). The molybdenum (VI)-thiocyanate complex is first formed in 0.02 M sulfuric acid. The anionic complex is extracted as an ion pair with tetraheptylammonium bromide (THAB) in 50 ml of toluene. This is back extracted into 50 ml methanol with 1,5-diphenylcarbazone (DPC), which displaces the thiocyanate, giving an intense red color which is measured at 540 nm. The detection limit was 2.5x10⁻⁸ M, with a sampling rate of 25 samples per hour.

Wetting Film Extraction of Mo(VI)

![Wetting Film Extraction of Mo(VI)](image-url)
SI Wetting Film Extraction Photometric Determination of Vanadium (IV) and Vanadium (V) [14]

The manifold in Figure-5 was used for the determination of vanadium (IV) and (V). The chelate of vanadium (V) with N-cinnamoyl-N-(2,3-xylyl)hydroxylamine (CXA), is extracted into benzene. The vanadium (IV) chelate extracts only at pH 5, but is nearly colorless. Hence, it is first oxidized to vanadium (V) with periodate and then extracted at pH 1.5. Introduction of 6 M hydrochloric acid following extraction resulted in maximum absorbance monitored in the benzene layer at 546 nm. Figure-6 shows the recorded signals for 0 to 300 ppm vanadium (V). The detection limit was 12 ng/ml, with a sampling rate of 15 per hour. The sensitivity is approximately 10 times higher than for manual extraction-photometric methods. Synthetic mixtures of 0.05 to 0.20 g/ml vanadium (IV) and vanadium (V) were accurately determined by this method.
SI Wetting Film Extraction Photometric Determination of Chromium (VI) and Chromium (III) [15]

The wetting film extraction system was applied for the direct spectrophotometric determination of chromium species in natural waters, using the manifold shown in Figure-7. The Cr(VI)-1,5-diphenylcarbazide (DCP) complex, formed at pH 1.6 in 0.012 M H₂SO₄, was extracted as the ion pair with perchlorate in an octanol/MIBK (1:2) film on PTFE Teflon. The film was eluted with 100 ml acetonitrile into a flow cell, and measured at 546 nm. Thiocyanate was as effective as perchlorate as an ion-pairing agent, but formed interfering complexes with some metal ions such as iron. The enrichment factor was 25. In the determination of Cr(III), it was oxidized with Ce(IV) at 45°C for 5 min prior to forming the DPC complex. The sequence of events was to load the sample and reagent into the reaction coil on the injection loop of value, V₂, for 56 sec, while aspirating water to wash the extraction coil (30 sec) and extraction solvent into the coil (4 sec). Air was aspirated for 2 sec, and then the sample was aspirated from the reaction coil for 120 sec. Finally, the elution solvent was aspirated from the injection loop in value, V, for 20 sec. Cr(VI) and Cr(III) were analyzed in tap water, lake water, and sea water. Spiked amounts of 10 to 75 mg/l were recovered, and the following amounts of Cr(VI)/Cr(III) were found in the samples: Tap water (3.5/4.2), lake water (<LOD, 9.2), sea water (<LOD, 6.0). The limit of detection (LOD) was 2.0 mg/l.

Cr(VI)/Cr(III) Manifold

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Figure-7
CONCLUSION

The wetting film extraction system is a convenient and efficient way of automatically extracting analytes for sample preparation and for direct spectrophotometric measurement. The model systems described illustrate that it is applicable to a wide range of chemistries and should find use in a variety of drug analysis methods. The amount of solvent waste is kept to a minimum, and measurements can be automated.
REFERENCES

ABSTRACT
D-Glucose equilibrates among the α-, β-anomers and its aldehyde form in water. The authors first devised the enzymic determination of α- and β-anomers of D-glucose by using β-D-glucose oxidase and mutarotase. Then, the half life of the equilibrium was estimated to be only 2.3 min at 37°C in blood. Concerning to anomic preferences of D-glucose-metabolizing enzymes, it became clear that hexokinase types I, II, III prefer to phosphorylate β-D-glucose to D-glucose 6-phosphate, while glucokinase prefers α-D-glucose. Aldehyde form of D-glucose is specifically reduced to sorbitol by aldose reductase. In the study of immunohistochemistry of glucokinase and mutarotase, it is revealed that glucokinase exists in nuclei of rat liver at low glucose concentration and diffuses to cytoplasm at high glucose concentration. Glucokinase exists in cytoplasm of rat Langerhans islets. Mutarotase localizes in nuclei of rat kidney and liver. α-D-Glucose preferentially stimulates insulin secretion from Langerhans islets, compared with β-D-Glucose. β-D-Glucose suppresses afferent activity of hepatic vagus nerve filaments of the guinea pig, and decreases stomach motility in the rat.

Key words: α-D-Glucose, β-D-Glucose, Aldehyde form of D-glucose, Hexokinase, Glucokinase, Mutarotase.
In water, D-glucose equilibrates among three structures, the α anomer (36.5%), β anomer (63.5%), and the so-called straight chain or aldehyde form (0.003%) as shown in Equation 1.

\[ \begin{align*}
\text{α-anomer} & \leftrightarrow \text{aldehyde form} \leftrightarrow \beta\text{-anomer} \\
(36.5\%) & \leftrightarrow (0.003\%) \leftrightarrow 63.5\%)
\end{align*} \] (1)

The anomerization half-life of either α- or β-D-glucose in pure water is 9.0 min at 37°C. In many biological systems, the interconversion among these three forms is catalyzed by mutarotase, phosphate ions, and other factors. Almost the same equilibrium of D-glucose is observed in human blood, and the anomerization half-life in human blood is only 2.3 min at 37°C. The mutarotational rate of D-glucose is so rapid in biological systems that the separate physiological chemistry of the α and β anomers and the aldehyde form of D-glucose could not be studied until methods for rapid enzymic determination of α- and β-D-glucose were established. In this report, enzymic micro assays and differences in metabolism and function of these three D-glucose structures are described.

1. Analyses of the α and β anomers of D-glucose

With regard to the determination of the α and β anomers of D-glucose, the authors devised a simple and specific micro determination of the α and β anomers of D-glucose with a β-D-glucose oxidase-mutarotase system in 1971.

The principle of the determination is shown in Equation 2.

\[ \begin{align*}
\alpha\text{-D-glucose} \rightleftharpoons \text{mutarotase} \rightleftharpoons \beta\text{-D-glucose} + \text{oxidase} \\
\beta\text{-D-glucose} \rightleftharpoons \text{O}_2 \rightleftharpoons \text{d-glucono-δ-lactone} + \text{H}_2\text{O}_2 \\
\end{align*} \] (2)

β-D-Glucose in the test solution (total D-glucose, 5 μg) is first oxidized to equimolar D-glucono-δ-lactone and H₂O₂ with equimolar consumption of oxygen in the presence of a sufficient amount of β-D-glucose oxidase. After the reaction is completed within only 50-70 s at room temperature, mutarotase (extracted from hog kidney) is added to the reaction mixture. The remaining α-D-glucose in the reaction mixture is changed to β-D-glucose quantitatively and is oxidized to give the additional formation of H₂O₂ and the additional consumption of oxygen. The first and second formations of H₂O₂ are measured by colorimetry in the presence of peroxidase and chromogen, or the first and second consumptions of oxygen are directly estimated with an oxygen electrode immersed in the reaction vial. From the two estimates of H₂O₂ or oxygen, one can then determine the ratio of α- to β-D-glucose in the reaction mixture.
The β-D-glucose oxidase used in these methods for determination of the α and β anomers of D-glucose is replaceable by β-D-glucose dehydrogenase, which is also specific for β-D-glucose. By the methods, the α and β anomers of free D-glucose in some animal tissues were analysed after rapid denaturation of glucose-metabolizing enzymes and mutarotase by treatment with a cold chloroform-methanol mixture. The percentages of the β anomers of free D-glucose in the liver, kidney, heart, blood, and plasma of rats were found to be 61.8, 61.0, 62.4, 62.7, and 62.9, respectively. The small differences between the above data and the percentage (63.5%) of the β anomer in an equilibrated D-glucose solution may be explained by the more rapid metabolism of β-D-glucose than α-D-glucose in these organs and blood.

2. Anomeric preferences of D-glucose-metabolizing enzymes

The authors partially purified hexokinase types I, II, III and IV (glucokinase) from rat liver, type I from rat brain and bovine retina, and type II from rat skeletal muscle. Hexokinase type II was also purified from mouse skeletal muscle and Ehrlich ascites tumor cells, and hexokinase types A and B were purified from chicken liver. Aldose reductase was purified from bovine lens.

D-Glucose anomeric preferences of these hexokinases were then studied. Three types (I, II, III) of hexokinase rapidly phosphorylate β-D-glucose as compared with α-D-glucose, while type IV (glucokinase) that exists exclusively in the liver and pancreatic islets phosphorylates α-D-glucose preferentially as compared with β-D-glucose. β-D-Glucose is more rapidly phosphorylated than α-D-glucose by hexokinase types I, II, and III (with low Km values) in almost all cells; and the product β-

<table>
<thead>
<tr>
<th>Source</th>
<th>Enzyme</th>
<th>β/α</th>
<th>Km α (mM)</th>
<th>Km β (mM)</th>
<th>Km aldβ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat liver</td>
<td>Hexokinase I</td>
<td>1.33</td>
<td>0.045</td>
<td>0.060</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>1.46</td>
<td>0.12</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>1.54</td>
<td>0.019</td>
<td>0.030</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IV (Glucokinase)</td>
<td>0.55</td>
<td>7.4</td>
<td>16.4</td>
<td></td>
</tr>
<tr>
<td>Rat brain</td>
<td>Soluble hexokinase I</td>
<td>1.45</td>
<td>0.029</td>
<td>0.048</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mitochondrial</td>
<td>1.44</td>
<td>0.041</td>
<td>0.056</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Latent</td>
<td>1.33</td>
<td>0.051</td>
<td>0.067</td>
<td></td>
</tr>
<tr>
<td>Rat skeletal muscle</td>
<td>II</td>
<td>1.53</td>
<td>0.14</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>Mouse skeletal muscle</td>
<td>II</td>
<td>1.48</td>
<td>0.136</td>
<td>0.270</td>
<td></td>
</tr>
<tr>
<td>EAT cells†</td>
<td>II</td>
<td>1.35</td>
<td>0.176</td>
<td>0.227</td>
<td></td>
</tr>
<tr>
<td>Chicken liver</td>
<td>A</td>
<td>1.51</td>
<td>0.094</td>
<td>0.197</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>1.52</td>
<td>0.093</td>
<td>0.159</td>
<td></td>
</tr>
<tr>
<td>Bovine lens</td>
<td>Aldose reductase</td>
<td></td>
<td></td>
<td></td>
<td>0.66</td>
</tr>
</tbody>
</table>

a. Km for aldehyde form of D-glucose; b. at 5 mM glucose; c. Ehrlich ascites tumor.
D-glucose 6-phosphate, is further dehydrogenated in the pentose phosphate cycle by β-D-glucose 6-phosphate dehydrogenase which is specific for the β anomer. On the other hand, α-D-glucose is more rapidly phosphorylated than β-D-glucose by glucokinase (with a high Km value) in the liver and pancreatic islets to form α-D-glucose 6-phosphate, which enters the pathway of glycolysis or is metabolized to glycogen. The aldehyde form of D-glucose in many tissues is reduced to sorbitol by aldose reductase, whose Km value for the aldehyde form is only 0.66 μM. Anomerization of D-glucose anomers is accelerated by mutarotase, and the mutarotational half-life of D-glucose anomers in the presence of a sufficient amount of mutarotase is less than a few seconds. The anomerization half-life of either α- or β-D-glucose 6-phosphate under physiological conditions is 1.5 s and the anomerization is accelerated by glucose 6-phosphate isomerase. Considering these anomic preferences of D-glucose-metabolizing enzymes, the metabolism of α- and β-D-glucose in higher animals is shown in Figure-1.

**Figure-1. Metabolism of D-glucose in higher animals**

* In the presence of mutarotase, t 1/2 is less than a few second.
GK, glucokinase; HK, hexokinase; PG-mutase, phosphoglucomutase.

3. **Anomeric preferences in D-glucose transport by animal tissues and cells**

From the point of glucose transport, animal tissues are classified into 2 groups: (1) insulin-independent tissues and (2) insulin-dependent tissues. β-D-Glucose was transported 1.2-1.7 times faster than α-D-glucose into insulin-independent tissues and cells such as rat brain cortex slices, retinas, and human red blood cells etc., except in the case of rat lenses.

On the other hand, in insulin-dependent cells such as rat diaphragms and fat cells (unpublished data), in which D-glucose uptake is stimulated 5-10 times by addition of insulin, no significant anomeric preference of D-glucose transport was observed.
It should be emphasized that β-D-glucose is incorporated more plentifully than α-D-glucose into not only insulin-independent (β-D-glucose preferential) cells but also insulin-dependent cells under physiological conditions, since the β/α anomer ratio of free D-glucose in blood is nearly the same as that of equilibrated D-glucose as described above.

It is difficult to study the anomeric preference of D-glucose transport by the liver, kidney, and small intestine, because high activities of mutarotase exist in these organs.

Table 2. Anomeric preferences of D-glucose uptake by animal tissues and cells

<table>
<thead>
<tr>
<th>1. Insulin-independent tissues and cells</th>
<th>Preference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human red blood cells</td>
<td>α &gt; β</td>
</tr>
<tr>
<td>Rat cerebral cortex slices</td>
<td>α &gt; β</td>
</tr>
<tr>
<td>Rat retina</td>
<td>α &gt; β</td>
</tr>
<tr>
<td>Rat Langerhans islets</td>
<td>α &gt; β</td>
</tr>
<tr>
<td>Rat lens</td>
<td>α = β</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2. Insulin-dependent tissue and cell</th>
<th>Preference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat fat cells</td>
<td>α = β</td>
</tr>
<tr>
<td>Rat diaphragm muscle</td>
<td>α = β</td>
</tr>
</tbody>
</table>

4. Cellular localization of glucokinase and mutarotase in higher animals

By an immunohistochemical technique, we examined recently changes in the subcellular distribution of rat liver glucokinase in response to external stimuli. Glucokinase immunoreactivity was found predominantly in the nuclei of hepatocytes. In situ perfusion of the liver with high glucose solution (20 mM) for 10 min caused a marked decrease in nuclear immunoreactivity and an increase in cytoplasmic immunoreactivity (Photo-1).

Under normal feeding conditions, glucokinase immunoreactivity was observed in both nuclei and cytoplasm of parenchymal cells. The nuclei were stained intensely and evenly, whereas the cytoplasm showed weak immunoreactivity of different degrees of staining intensity depending on the location of the cells. After 72 h of fasting, glucokinase immunoreactivity was markedly decreased in all hepatocytes. After the start of refeeding, the cytoplasmic immunoreactivity began to increase. After 10 h of refeeding, strong immunoreactivity was observed in both the cytoplasm and the nuclei of all hepatocytes, and appreciable glucokinase immunoreactivity was detected at the plasma membrane of some hepatocytes.

Subcellular distribution of glucokinase in rat liver during postnatal development was examined immunohistochemically. Before day 11 after birth, only some hepatocytes were immunostained, in the cytoplasm but not in the nucleus. From day 15, at which time a dietary change from milk to laboratory chow begins to take place, glucokinase immunoreactivity increased;
this increase was associated with increases in glucokinase activity and in glucokinase protein, and also the immunostaining was observed mainly in the nuclei. The results indicate that dramatic changes in the distribution of glucokinase in developing rat liver may be related to dietary change.

Rat liver is known to contain a regulatory protein (RP) that inhibits glucokinase competitively with respect to glucose. By an immunohistochemical technique and by cell fractionation in glycerol, we investigated the distribution of RP in rat liver and its changes in response to high glucose. RP was detected almost exclusively in the nuclei of the parenchymal cells of both fed and fasted rats. In situ perfusion of the liver with 20 mM glucose for 10 min caused a marked decrease in nuclear RP and an increase in cytoplasmic RP. These results indicate that RP is present predominantly in the nuclei of hepatocytes and is translocated from the nucleus to the cytoplasm in response to high glucose.

The authors also found that the four forms of mutarotase from rat kidney reacted with antibody against mutarotase type II from rat kidney and no spur formation was observed in the double immunodiffusion test. This result indicates that all of these forms of mutarotase have the same antigenic determinants. The localization of mutarotase in rat kidney was investigated by fluorescein-
labelled and peroxidase-labelled antibody technique, and by the method of isolation of the nuclei and cytoplasm with non-aqueous solvents. In these immunohistochemical studies, mutarotase was almost exclusively found in the nuclei of epithelial cells of renal tubules, glomeruli and mucosal cells of the small intestine, and of liver parenchymal cells of the rat. These results suggest that mutarotase may be involved in the metabolism of D-glucose in the nuclei.

Mutarotase activity was not changed in the liver, but was markedly decreased in the kidney when nephrotic syndrome was induced in rats by the intravenous injection of rabbit anti-rat kidney serum. Mutarotase activity in the serum and urine of normal subjects and patients with known renal diseases was also determined with our mutarotase assay using an oxygen electrode and \( \beta \)-D-glucose oxidase. Elevated urinary mutarotase levels were observed in 7 of the 8 patients with nephrotic syndrome.

5. Different physiological functions of the \( \alpha \) and \( \beta \) anomers of D-glucose\(^{1,4} \)

1) Secretion of insulin from \( \beta \) cells of Langerhans islets of the rat\(^{1,11} \)

The authors studied the effects of D-glucose anomers on insulin secretion in isolated Langerhans islets of the rat, because the islet is one of the best tissues for sensing the concentration of blood glucose. Isolated islets (10-20) were incubated with \( \alpha \)-, \( \beta \)- or equilibrated D-glucose (2 mg/ml) at 37°C for 5 min, and the insulin secreted was measured by radioimmunoassay. The amounts of insulin secreted during the incubation with \( \alpha \)-, \( \beta \)- and equilibrated D-glucose were 15.0, 8.0, and 9.9 \( \mu \)units per 10 islets, respectively. Thus, insulin secretion stimulated by the \( \alpha \) anomer was nearly two times higher than that by the \( \beta \) anomer. Considering the rapid mutarotation of one anomer to the other during the 5 min incubation, the real difference between the effectiveness of \( \alpha \)- and \( \beta \)-D-glucose on insulin secretion may be much larger than two.

Pseudo \( \alpha \)- and \( \beta \)-DL-glucose in which the ring-oxygen atoms of \( \alpha \)-DL- and \( \beta \)-DL-glucopyranose molecules are displaced by methylene groups were used by the authors as synthetic analogs of glucose anomers to study the mechanism of D-glucose-stimulated insulin release by pancreatic islets. Neither isomer was phosphorylated by liver glucokinase, whose properties are quite similar to those of islet glucokinase, nor stimulated insulin release from islets. The \( \alpha \) isomer, but not the \( \beta \) isomer, inhibited both glucose-stimulated insulin release (44% inhibition at 20 mM) and islet glucokinase activity (36% inhibition at 20 mM) in a concentration-dependent manner and to a comparable degree.

Glucokinase and insulin-releasing activities in the rat pancreatic islets which were incubated at 37°C for 5 min with alloxan (100-500 \( \mu \)M) or ninhydrin (10-100 \( \mu \)M) and then washed with buffer were measured. Alloxan (500 \( \mu \)M) and ninhydrin (100 \( \mu \)M) inhibited both activities by about 80%.

These results provide additional strong evidence for the essential role of islet glucokinase in glucose-stimulated insulin release.
2) Suppression of afferent activity of hepatic vagus nerve filaments of the guinea pig

The authors studied the glucose effect on afferent discharges recorded from the nerve filaments dissected from the hepatic branch of the vagus nerve of the guinea pig. Recordings were made from isolated and perfused liver preparations and in vivo to study the effect of the $\alpha$ and $\beta$ anomers of D-glucose on the afferent discharge rate. Both infusions of these anomers (30 mg or 50 mg dissolved in 10 ml D-glucose-free Ringer solution) into the portal vein in perfused liver preparations and intraportal injections of these anomers (50 mg dissolved in 0.2 ml saline) in vivo partially suppressed the afferent activity. However, the effect of the $\beta$ anomer on suppression was stronger than that of the $\alpha$ anomer. The results indicate the existence of anomeric stereospecificity of D-glucose action on the hepatic "glucose sensor".

3) Decrease of stomach motility in the rat

The injection of $\alpha$-, $\beta$-, or equilibrated D-glucose solution into the cranial side of the carotid artery decreased gastric pressure caused by insulin in rats with bilateral adrenalectomy. This effect was not reproduced after vagotomy at the cervical level. The effect of $\beta$-D-glucose was more rapid and potent than that of $\alpha$-D-glucose. The injection of isotonic NaCl solution, however, produced no change in pressure.

All of the above results are listed in Table 3.

Table 3. Anomeric preferences of D-glucose-recognizing cells of higher animals

<table>
<thead>
<tr>
<th>Preference</th>
<th>1. $\alpha$-D-Glucose preference (paraneurons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preference</td>
<td>Taste bud cells (sweetness of D-glucose)</td>
</tr>
<tr>
<td>Preference</td>
<td>Pancreatic islets ($\alpha$ cells, glucagon)</td>
</tr>
<tr>
<td>Preference</td>
<td>Pancreatic islets ($\beta$ cells, insulin)</td>
</tr>
<tr>
<td>Preference</td>
<td>Small intestine (glucagon-like materials)</td>
</tr>
<tr>
<td>Preference</td>
<td>$\alpha &gt; \beta$</td>
</tr>
<tr>
<td>Preference</td>
<td>$\alpha &gt; \beta$</td>
</tr>
<tr>
<td>Preference</td>
<td>$\alpha &gt; \beta$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Preference</th>
<th>2. $\beta$-D-Glucose preference (neurons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preference</td>
<td>Glucose-sensitive brain neuron (inhibition of stomach motility)</td>
</tr>
<tr>
<td>Preference</td>
<td>Hepatic vagus nerve (glucose sensory activity)</td>
</tr>
<tr>
<td>Preference</td>
<td>$\alpha &lt; \beta$</td>
</tr>
</tbody>
</table>

4) Bacteria

The effect of D-glucose anomers on the germination of dormant spores of Bacillus megaterium QM B1551 was studied by the authors. $\alpha$-D-Glucose (1 mM) elicited a slight germinative response of dormant spores during a 10 min incubation at 37°C, while about 60% of the dormant spores germinated with $\beta$-D-glucose (1 mM) under the same conditions. We also found that only a
negligible amount of β-D-glucose is taken up by the dormant spores. These observations suggest that a β-D-glucose-stereospecific receptor site for the germination exists on the surface of the dormant spores of the bacillus.

The effect of α- and β-D-glucose on the respiration of dormant spores, germinated spores, and vegetative cells of *Bacillus subtilis* and *B. megaterium* was also studied. The net amounts of oxygen consumed per min by $10^{10}$ germinated spores of *B. subtilis* after the addition of α- and β-D-glucose were 1.6 and 6.6 μg ($β/α = 4.13$), while those by *B. megaterium* were 4.5 and 6.8 μg ($β/α = 1.51$), respectively. However, the net amounts of oxygen consumed by vegetative cells per min after the addition of α- and β-D-glucose were identical: 443 μg for *B. subtilis* cells and 604 μg for *B. megaterium* cells.

6. Studies on anomers of D-mannose, D-galactose, and D-fructose

α-D-Mannose was found to be more effective than the β anomer in stimulating insulin secretion from the perfused rat pancreas. A method for analysis of α- and β-D-glucose using β-D-galactose dehydrogenase and mutarotase was devised. It was found that α-D-galactose is more easily incorporated into rat lens than the β anomer, probably due to the existence of α-D-galactokinase in the lens. Fructokinase from bovine liver is specific for the β-furanose form of D-fructose.

REFERENCES


PHARMACEUTICAL EDUCATION AND ACCREDITATION IN THE USA

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ABSTRACT
The objectives for this presentation are to trace the historical development of pharmaceutical education in the United States during this century and to discuss changes occurring and contemplated as we enter the 21st century. The perspective for this discussion will be the accreditation program of the American Council on Pharmaceutical Education, the agency for the accreditation of professional degree programs in pharmacy and providers of continuing pharmaceutical education. To develop this perspective, the background, value and purpose of accreditation are sketched, and the procedures and process for setting standards for accreditation are outlined. The progressive development of pharmaceutical education is related to major studies presenting the need for changes and their influence upon the adoption of accreditation standards and their subsequent implementation. A possible model for the establishment and administration of international educational and competency standards based upon adoption of the principles of accreditation is suggested.

PHARMACY EDUCATION AND ACCREDITATION IN THE USA
The progression of pharmaceutical education in this century and as we soon enter the 21st century is, for purposes, of this discussion, treated from the perspective of accreditation: a private system utilized for public purposes. To develop this perspective, the background, value and purposes of accreditation are presented and the procedures and processes for setting standards upon which accreditation is based are described. At the conclusion of this discussion, a possible model for establishing international educational and competency standards is offered that adapts and applies the general principles of accreditation.
Accreditation: Purpose, Process and Values

Accreditation is a voluntary, non-governmental peer review process long accepted in the United States, but is generally unknown in most other countries that rely upon governmental supervision or central regulation and control of the educational systems. Accreditation, by definition, means the process whereby an agency grants public recognition to an institution of higher learning or a program of higher learning that meets certain established qualifications and educational standards, as determined through initial and periodic evaluations.

Both on the continent of Europe and in England, the history of higher education has involved the redirection of the universities, many of which were founded in the Middle Ages. In contrast, the record in America has been quite different. In the United States, with only an indirect heritage from the medieval university, but with an immediate dependence upon the Protestant university traditions of England and Scotland, the imprint of geography and economics, as well as that of religion and politics, was indelibly inscribed first on our colleges and later on our universities, as they were freshly created in a manner distinct from, even though related to, their earlier prototypes. In no feature is this more apparent than in the area of control of academic standards, where the system in American has been developed legally and educationally on the basis of community control. With the adoption in 1791 of the 10th Amendment to the U.S. Constitution, education, as one of the powers not delegated to the federal government and not prohibited to the states, was considered to be a state and local responsibility. Accordingly, at the federal level of government, there is no central officer with responsibilities and authorities for educational standards in the sense generally attributed to a minister of education, nor is there a system of external governmental examinations. In the absence of any such direct federal controls, accreditation subsequently emerged as a voluntary system for the establishment and maintenance of standards.

By historic design, therefore, education in the United States functioned with few restrictions. However, in the diversity of the system, weaknesses also became apparent, and accreditation, the American form of control over academic standards and processes, emerged as the means
for assuring better education to the public, and of protecting against the pretentious, shoddy, and even fraudulent institutions which often called themselves colleges or universities. The circumstances were particularly noteworthy, and dangerous, in the case of the professions and professional education. The chaotic conditions of the earlier period of American education prompted Woodrow Wilson [1] then president of Princeton University (later the 28th President of the United States) to proclaim in 1907: "We are on the eve of a period of reconstruction. We are on the eve of a period when we are going to set up standards. We are on the eve of a period of synthesis, when, tired of this dispersion and standardless analysis, we are going to put things together into something like a connected and thought-out scheme of endeavor." The accreditation movement then progressed for institutional and professional accrediting agencies throughout the first half of this century, to serve as the balance between institutional autonomy, independence and freedom, and the institution's or program's responsibilities to students, the public, the professions, and various levels of government.

As a self-regulatory process, accreditation includes certain rights and responsibilities for both institutions and accrediting bodies. Coupled with these institutional rights and responsibilities are the rights and responsibilities of accreditation that keep institutions responsive and insure that they provide education of quality. The nongovernmental accreditation system is key to ensuring educational integrity and providing public confidence in the quality of the institution or institutional program. In a real sense, accreditation serves as the conscience of higher education.

For purposes of clarification, it should be noted that the U.S. Secretary of Education, a member of the President's cabinet, has a role in educational standards, but that role is statutorily circumscribed. The U.S. Secretary of Education has legal authority only to recognize or approve non-governmental accrediting agencies who set and administer their own standards, such as the American Council on Pharmaceutical Education. This recognition process is rigorous and the criteria, particularly in recent years, have had an influence on the educational process. The criteria for Secretarial recognition serve as the basis for a
determination by the Secretary that an accrediting agency is a reliable authority as to the quality of education offered within the scope of that agency's activity. In turn, the federal government relies upon recognized accrediting agencies for decisions regarding the investment of public funds. For example, to be eligible for the award of federal grants, including construction grants and student loans and scholarships, an institution or program needs to be accredited by an agency recognized by the U.S. Secretary of Education.

Importantly, accreditation in the professions, including pharmacy, is supported by licensure, the legal basis for practice, in that State licensing bodies, such as Boards of Pharmacy, use accreditation in their decisions with regard to appropriate or approved educational standards, as well as with regard to those colleges of pharmacy's programs that satisfy such standards. Generally, each Board of Pharmacy adopts, annually, the American Council on Pharmaceutical Education's standards as being those of the Board, and the individual programs of the colleges of pharmacy listed in the Council's Annual Directory of Accredited Professional Programs of Colleges and Schools of Pharmacy, are approved. In this manner, authority and responsibility for standards, and approval of the individual programs that meet these standards, rightfully remain with the Boards of Pharmacy. The interrelationship between accreditation and licensure is also germane to continuing pharmaceutical education. The accreditation program for continuing pharmaceutical education is also accepted by state Boards of Pharmacy to serve as a basis for satisfying continuing education re-licensure requirements that may exist.

The values of accreditation are many and the American Council on Pharmaceutical Education's accreditation program serves concurrently several constituencies: the public, students and prospective students, the college of pharmacy, the parent institution, and the profession. First, let us consider the values to the public. Accreditation provides assurances of external evaluation of the professional program in pharmacy by a responsible agency recognized by the U.S. Secretary of Education, and offers a finding that there is conformity to the general expectations of the profession. These factors constitute a major public reason for accreditation. Colleges of Pharmacy, and their parent institutions, enjoy autonomy in their
governance and operation. However, they hold a public trust with a quid pro quo: namely, that society expects and deserves accountability for its educational enterprise.

An additional public consideration is that of improvement in the professional services available from pharmacy practitioners, since accredited programs are expected to modify their curricular requirements to reflect changes in technologies, knowledge, and skills necessary to develop professional competencies of students. Curricular content placed within the confines of a legal statute is likely to become ossified, whereas accreditation is a dynamic process that readily accommodates change. Indeed, accreditation has a special responsibility to incorporate and reflect the evolving state of the art and science of the profession. This requires a synthesis of the perspectives of the educator and practitioner. The public expect the American Council on Pharmaceutical Education, as the accrediting agency in pharmacy, to establish meaningful, relevant standards for the educational preparation of pharmacy practitioners, and for their continued currency and competence. Yet another value to the public is that of a decreased need for intervention by governmental agencies in the operation of educational institutions, since through accreditation, the institution provides privately for assurances and enhancements of educational quality. Accreditation is also relied upon by private foundations, as well as by governmental agencies, as a basis for investing private and public moneys, respectively.

Accreditation is of immense importance to students and prospective students. An accredited program offers assurances that the educational program they intend to pursue, or are, in fact, pursuing, has been found to be satisfactory through peer review, and will meet their educational needs. Accreditation is a fundamental prerequisite for entering into the profession, in view of the linkage previously described between licensure and accreditation in pharmacy.

Moreover, accreditation is of critical importance to Colleges of Pharmacy and their parent institutions. The accreditation process involves self-evaluation and self-directed program improvement. No matter how dedicated to quality performance, the surfactant of outside assessment and validation of self-analysis is extremely valuable. Of no small importance to
both the institution and the College of Pharmacy is their own enhanced reputation owing to the public's high regard for accreditation. It is noteworthy that standards for accreditation not only insure the educational quality of professional degree programs in pharmacy and assure educational preparation for pharmacy practice, but also, foster the collegiate infrastructure necessary to effective graduate and research programs. Such standards relate to the adequacy of faculty resources, the relationship between faculty quality and research or faculty development programs, provision for teaching and research laboratories, basic laboratory equipment and academic and administrative computing capabilities, and access to adequate library holdings and drug information resources.

Lastly, accreditation is of critical importance to the profession itself. Accreditation provides a means for the participation of practitioners in setting the requirements for preparation to enter the profession and to maintain competency, since the process of accreditation provides a mechanism for formalizing interaction between academic programs and practitioners. This blending of perspectives and the balancing of different viewpoints among the entire community of interests is beneficial to the profession. Moreover, practitioners can be assured that those who follow after them will be adequately prepared for the current as well as future responsibilities of pharmacy practice. The philosophy of the American Council on Pharmaceutical Education is that accreditation is, foremost, an improvement process, for the profession and public it serves.

**Historical Development of Pharmaceutical Education and Accreditation Standards**

Medicine and law were the first professions to institute professional accrediting bodies as a mechanism for providing quality assurances and to raise the educational standards in their respective fields. In 1910, Abraham Flexner, the important medical reformer, issued the classic Flexner Report on Medical Education in the United States and Canada [2]. This report presented the need for new and uniform standards for medical education. Implementation of these standards revolutionized both medical education and medical practice. The successful example of medicine, and then law, affected other academic specialties at a time when
conditions were ripe for the spread of this native method of establishing and maintaining standards. The accreditation movement in the professions progressed in the 1920's and early 1930's, leading to the subsequent developments of accrediting in the fields of dentistry, nursing, veterinary medicine, and pharmacy.

Development of the first accreditation standards was initiated shortly after the founding of the American Council on Pharmaceutical Education in 1932, and were finalized in 1937 [3]. Since that time these evaluative criteria have been revised periodically, about every six years, in keeping with changes occurring and contemplated in pharmacy practice and pharmaceutical education. The present accreditation standards were adopted in June 1997, following a revision process that was begun in September 1989. It is anticipated that effective dates for these newly adopted standards will be set for June 2000 in accord with a transitional schedule for implementation. The initial standards, and all subsequent revisions as proposed throughout our history, have taken into account relevant studies, needs and trends. The revision processes have taken place in keeping with published policies and procedures which call for advance notice of proposed changes and opportunity for comment, including open hearings, prior to adoption and implementation.

Major factors which influenced the initial and subsequent revisions of standards may be traced by review of key studies of the pharmacy profession in the United States, since these included analyses and recommendations and represented the collective pulse of the time.

The Pharmacy Curriculum Study of 1927

The first major study of pharmacy in this century, and one critical to the formulation of the first accreditation standards, was the Pharmacy Curriculum Study of 1927 [4]. This study was conducted by Dr. W. W. Charters, a non-pharmacist, and Dean of Education at the University of Illinois, along with the assistance of a pharmacy advisory committee. The "Charters Study", was a publicly-oriented analysis initiated from concerns stemming from the lesser role and rank
given to pharmacists in the armed services during World War I. The Surgeon General's low opinion concerning the pharmacist as a professional was influenced, ironically, by the negative impressions of pharmacy, and particularly, pharmacy's educational requirements, as presented by Abraham Flexner, author of the Flexner Report, cited earlier. The curriculum study of Charters conducted during the period 1923 to 1927, recognized pharmacy as noble and honorable profession. It's findings stimulated sweeping changes in pharmaceutical education and pointed pharmacy practice in a new direction. The Charter's study related pharmacists, as professionals, to the quality of their educational preparedness for practice. The study uncovered the broad health care activities and responsibilities of the pharmacist, who was seen as an autonomous health care practitioner with patients who have various health needs, and were accountable to the public served. Professional activities, as seen by Charters, were grounded in a humanist practice and in a social context. Practice needs, therefore, dictated an educational base that needed to be highly sophisticated. As a result of this study, essentials for pharmaceutical education were codified, and the progression of educational standards that followed have reflected such milestones of curricular evolution as the 2-year program leading to the PhD, the 3-year program leading to the PhC, and the 4-year program leading to the baccalaureate in pharmacy degree, that replaced the previous educational programs. The first accreditation standards called for a 4-year program leading to the baccalaureate in pharmacy degree and that degree only. A college degree as entry-level educational preparedness, also resolved a previously debated issue of whether or not graduation from secondary school was needed for admission to the study of pharmacy.

The Pharmaceutical Survey of 1946

In 1946, the Pharmaceutical Survey [5] was conducted under the leadership of Edward C. Elliot, former president of Purdue University. This survey provided a blueprint for the advancement of pharmacy practice and its findings are critical to the subsequent and continuing development of pharmaceutical education. The membership of Elliot's committee included the pharmacy leaders of the day and benefited from the continued service of W. W. Charters. The
The intent of the study was to develop proposals and designs for the progressive betterment of pharmacy as a profession. The report, published in 1948, acknowledged the previous work of Charters, and lamented its incomplete progress, suggesting that had its spirit and recommendations been more widely followed, the development of pharmaceutical education in the United States would have been accelerated. The Elliot report recommended immediate improvement in the then 4-year baccalaureate in pharmacy program, based upon expansion and refinement of the biomedical and pharmaceutical sciences. Major weaknesses were found to exist in the biological sciences and pharmacology, as well as in pharmacy administration. General education was also found to be in need of strengthening, not to serve as a frill or embellishment, but rather, to support the essential aspects of the professional studies.

The limitations of the then 4-year baccalaureate in pharmacy program became apparent when consideration was given to the great advances in scientific information and to the development of new medical substances, their distribution and therapeutic application. Whereas it was felt that a 5-year program could overcome some of the limitations observed, it was considered to be an inadequate solution to the perceived problem. The findings of this 1948 survey stated that "in light of the evidence available it appears that a 6-year program for pharmaceutical education would be highly desirable." The reasons offered in support of this conclusion involved advantages to be gained by both the expanded curricular content and the public values associated with the professional doctoral degree. This recommendation for a 6-year program was made with the stated awareness that this would be a goal to be achieved in time. The Elliot Report, therefore, looked to a transitional period wherein the doctor of pharmacy program would steadily evolve. Accreditation standards continued to be revised within the 4-year curricular framework, but attentional emphasis was given to science areas within the confines of the existing curricular boundaries. However, the Elliot Report launched discussions by educators and practitioners alike regarding extension of the curriculum, a discussion that was to persist throughout the remainder of the century. An interim compromise was struck in the mid-1950's, in which a 5-year baccalaureate in pharmacy program would
replace the previous 4-year program. Accreditation standards reflecting this change became effective in 1960, with the first graduates in 1965.

Study Commission on Pharmacy

The Report of the Study Commission on Pharmacy [6] conducted from 1973 to 1975, was chaired by Dr. John S. Millis, former president of Case Western Reserve University. This report laid out a broad philosophical system by which the profession could establish long-range educational policies. A key recommendation of the Millis Report was that the professional curriculum should be based upon competencies desired for graduates, rather than upon the basis of knowledge available in the several relevant sciences, thereby changing the approach to curricular study. The recommendation was also made that colleges of pharmacy with adequate resources be encouraged to proceed to the 6-year doctor of pharmacy program as their professional program offering. The philosophical underpinnings of the Millis Report were applied to the accreditation process in terms of guidelines for administration, particularly curricular construction based upon established competencies.

Task Force on Pharmacy Education

The Report of the Task Force on Pharmacy Education, commissioned by the American Pharmaceutical Association, was released in 1984. Dr. John C. Weaver, former president of the University of Wisconsin System chaired this Task Force [7]. This report examined the functions and responsibilities of present and future pharmacists. The report endorsed the accreditation standards under consideration during the early 1980's whereby a common core of education and training was coupled with options for differentiation. In addition, the Task Force recommended that a 6-year doctor of pharmacy program "evolve as the desired goal with the intent that it become the sole entry-level for the practice of pharmacy." Moreover, it recommended that accreditation standards along these lines be in place by 1990.
The accreditation standards that were adopted in 1985 and have continued until the adoption of revised standards in June 1997, reflected the several studies cited and represent a consensus of professional and public viewpoints as determined through the revision processes. As standards were formulated, the findings and recommendations of these and other studies were considered, adjusted and modified, in keeping with the opinions and positions of the large community of interests affected by the accreditation process. Final considerations attended to and balanced competing interests and concerns.

It can be observed that, in the first part of this century, pharmaceutical education in the United States achieved uniform standards, and then proceeded to improve the educational process in an evolutionary manner, yet setting the stage for any necessary improvements. The continuous and overarching issue was to assure educational preparedness for present and future requirements for practice. A summarization of the status of pharmaceutical education was presented in a 1952 report on curriculum by Blauch and Webster [8]. It was stated that during the first half of the 20th century, pharmaceutical education in the United States moved from a chaotic condition to a fairly well-organized and standardized system. Moreover, it stated that through the half-century, the steps forward were usually opposed, but in spite of this, progress was made. These authors further indicated that while the designing of a curriculum is essentially the responsibility of the faculties of the colleges and schools of pharmacy, guidance from external bodies was helpful to set general patterns and correct deficiencies. An increasingly important role for accreditation was forecast to promote progress in pharmaceutical education.

New Accreditation Standards for the Turn-of-the-Century

The Council has continuously provided the profession the forum of the revision process for dialog regarding the improvement of educational preparedness for practice. In September of 1989, the American Council on Pharmaceutical Education once again indicated that it was time for the customary and periodic review of the standards in effect. Advance notice of the
intention to propose revisions was given, along with a presentation of the mission of the pharmacy practitioner as being the assumption of responsibility for providing pharmaceutical services that ensure rational drug use in the care of patients. Furthermore, it was stated that the goals of the pharmacy practitioner's services include the provision of drug therapy that is appropriate, safe, efficacious and cost-effective, the education and motivation of patients to assume an appropriate and active role in self-care and in the management of their drug therapy as related to their particular medical conditions; and effecting the appropriate distribution of medication to patients. Based upon the Council’s evaluation of practice developments, including future challenges and the corresponding educational process needed, the revised standards were to proposed in a manner that would converge existing programmatic standards for the baccalaureate in pharmacy and doctor of pharmacy standards and focus upon a single professional program in pharmacy leading to the doctor of pharmacy degree. Importantly, the revision process signaled the opportunity for comprehensive curricular reconstruction and reformation. The Council’s opinion regarding the degree program framework as well as other revisions to be proposed were presented with the understanding that full and open discussions would be held, in accord with the system for revision historically and successfully employed in the past.

A standards revision procedure and schedule, issued in 1990, presented a step-wise procedure for development and implementation to be conducted over a ten-year period. The revision process provided opportunity for the entire community of interests: educators, students, practitioners, employers, and boards of pharmacy, to turn critical issues over in the collective mind so as to insure that our responsibilities to the future would be discharged. Long-standing educational issues, including the issue of the 6-year doctor of pharmacy degree program as presented by the Elliot Report, were offered up for review within the construct of dialog surrounding the periodic revision of accreditation standards. A doctor of pharmacy curriculum was to be studied on the basis of specific competencies and curricular content needed for a generalist pharmacy practitioner, in all practice sites. This revision process was begun with an awareness that we were approaching the 21st century on the cusp of profound changes in
health care delivery and health care systems, and were facing the educational and professional challenges associated with these changes. To meet these challenges, the structure of pharmaceutical education needed to be changed. Trends in health care, health care financing and the affect of modern biology and biotechnology on drug therapy and delivery systems, were sensed to soon impact dramatically on practice and education. Hospitalized patients, it was correctly forecast, would go home quicker and sicker. The factor of the growth in the elderly population of America and the impact upon drug utilization were noted. Self-care and a shift to non-prescription drugs were considered as key factors. Pharmaceutical services in the community would expand along with expectations to improve patient outcomes and reduce costs of care. This would require the pharmacist to detect, resolve and prevent drug related problems that can lead to drug related illnesses. The expectations placed upon practitioners, especially in the community, would become increasingly demanding, and require the seamless provision of distributive, clinical and information services. The advent of managed care and the booming growth of health maintenance organizations would further impact the pharmacy landscape. A new body of evidence now reveals both the problems of drug misadventuring and the value of pharmaceutical care. Schumock and colleagues [9] have presented information pertinent to the value of the clinical practice of pharmacy. Johnson and Bootman [10], report preventable drug-related morbidity and mortality and the costs to society of the misuse of prescription medications. Clearly, the challenges seen some time ago are a part of the here and now, and can be expected to loom larger in our future. However, the future is bright, since the pharmacy profession in the United States is now positioned to respond based upon a responsible and responsive educational foundation.

The new accreditation standards, adopted in June 1997, rest upon historically developed standards, yet respond to new educational and professional qualities needed for general practice in any practice setting. Professional competencies are presented along with expected curricular content. The new standards are concerned not only with what is presented, but also, with how it is taught and with evidence that it is learned. Wherever appropriate or possible, each standard supports an interplay between educational processes and outcomes, thereby
assuring that process improvement and outcome assessment are a part of the culture of pharmacy education. Outcomes are expected to be documented and evidence should exist that evaluative information is systematically applied for purposes of continuous improvement, in accord with established concepts of total quality management. The mission and goals of the college of pharmacy should be interwoven with other program elements, being fundamental to programmatic planning, development and implementation. The mission and goals also provide the framework for assessment of effectiveness. The organizing principle for the curriculum should be pharmaceutical care as considered in the pharmaceutical literature and as presented by Hepler and Strand [11] and in the Report of the Commission to Implement Change in Pharmaceutical Education [12]. A key aspect of the standards address issues of teaching, learning and evaluation, including teaching strategies that deal with the development in the student of oral and written communication skills and problem solving abilities. Examinations and testing are expected to condition students for critical thinking rather than for short-term retention or memorization of specific details or isolated facts. Last, but not least, are standards related to the professionalization of students, calling for faculty mentoring in academic pursuits as well as for nurturing a positive attitude about the profession and the provision of pharmaceutical care.

In summary, it may be said that the newly revised standards involve renovation and reconstruction. However, these standards can only serve as the blueprint for our new educational endeavor. It is the faculties of the colleges of pharmacy who, through their creativity and innovation, will ultimately shape the future of pharmaceutical education.

Application of Accreditation: A Possible International Model

The principles of accreditation that embrace a non-governmental, profession-based quality control system suggest a possible model for the establishment and administration of international educational and competency standards. Modification and adoption of the fundamental basis of accreditation, particularly, the broad participation that generates
standards and the profession's ownership of the standards so formed, may offer a means of fostering educational and competency standards that may be accepted and utilized by the international pharmacy community. Moreover, these principles may be among ways and means to accommodate the complexity of myriad international laws and regulations that govern our field. The development of what shall be termed, the "INTERACCRED" network, may enable the formulation of accepted international standards that support the potential for their interchange among the geopolitical jurisdictions. Ministries of health and education, or other governmental bureaus, may then rely upon this network, in much the same manner as do the 51 Boards of Pharmacy in the United States and Puerto Rico, each with their separate laws and regulations. This network could be utilized in governmental decision making processes, while continuing to satisfy their own specific legal requirements. The administrative component of this model is envisioned as remaining with each geopolitical jurisdiction. Having once mutually developed and subsequently subscribed to a set of international educational and competency standards, the process for evaluation against these standards would follow an agreed upon protocol by each participant of the "INTERACCRED" network, with reciprocal acceptance of accreditation decisions. While the serrated edges of this idea need to be smoothed, learning of accreditation's capabilities capturing the proven strengths and adapting accreditation for the establishment of international standards, may well be worth the effort required. The global village we now inhabit requires new thinking by which we can foster and accommodate the many changes that abound. Thank you.
REFERENCES

CONTROL OF LIPOGENESIS
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ABSTRACT
This review of the central themes in the control of lipogenesis in mammals emphasizes the maintenance of caloric homeostasis during the diurnal feed-fast cycle. Long-term regulation is executed principally by the hormones insulin and glucagon (during feeding and starvation respectively) by signalling induction and repression of a set of lipogenic enzymes catalyzing the synthesis of fatty acids and fat (triacylglycerol or TAG) from precursors that can form acetyl CoA. Short-term control mediated by insulin and glucagon affects the state of phosphorylation of a second (overlapping) set of enzymes through the action of protein kinases and protein phosphatases. In general, feeding and the accompanying release of insulin bring about the dephosphorylated mode of this enzyme set thereby promoting the flux of organic precursors into the major storage metabolic fuels: glycogen in liver and TAG in liver and adipose tissue. In progressive starvation, the phosphorylated enzyme mode is established causing the release of glucose from glycogen, lipolysis of TAG (release of free fatty acids) and gluconeogenesis from amino acids (in liver). The roles of cyclic AMP protein kinase and the recently characterized 5'AMP-activated protein kinase are discussed vis-à-vis protein phosphatases in mediating glucagon and insulin signalling, in particular, the key enzymes acetyl CoA carboxylase (fatty acid synthesis) and HMG CoA reductase (cholesterol synthesis). Newly described feedback controls of appetite and uncoupled metabolic fuel expenditure by adipose tissue further limit TAG accumulation.

KEY WORDS: Lipogenic enzymes, Acetyl CoA carboxylase, HMG CoA reductase, Protein kinases, cAMP-protein kinases (PKA), 5'AMP-activated protein kinase (AMPK), (AMP kinase kinase) (AMPKK), Protein phosphatases, Metabolic control mechanisms
INTRODUCTION

Broadly defined lipogenesis should include the biosynthesis of (a) essential lipid components in the cell, predominantly the fatty acids and cholesterol needed for cellular growth and membrane turnover; (b) certain intracellular lipid signal molecules such as inositol triphosphates [1], ceramides [2] and fatty acyl CoA [3], and the major caloric metabolic fuel, triacylglycerol (TAG), reviewed here in the context of animal fat reservoirs.

In most natural settings, mammals consume food stuffs only at intervals interspersed with variable periods of relative starvation during which metabolic demand (ATP turnover as indexed by respiration) is met by utilizing the storage fuels on board, first liver glycogen, then adipose TAG, and in long-term starvation muscle protein. Thus, replacement synthesis of glycogen and TAG is primarily linked to the input of food, facilitated by the release of insulin into the circulation. During starvation glycogenolysis in liver provides glucose, and lipolysis of TAG in adipose tissue generates free fatty acids, both events signalled by the elevation of circulating glucagon (drop in the insulin/glucagon ratio). The limited supply of glucose from glycogen is later supplemented by gluconeogenesis from amino acids transported to liver, since glucose is always required for oxidative metabolism by certain tissues, especially the nervous system. Indeed elevated free fatty acids is the preferred fuel in starvation for some tissues since it spares the consumption of glucose.

Beyond the strategy for conservation of acquired metabolic fuels by the organism, maintenance of caloric homeostasis in the blood, even during the extremes of the starvation-feeding cycle, is achieved by signalled changes in the fluxes of metabolites into and from the primary storage depots. For example, during feeding, the oversupply of glucose is blunted by entrapment in liver glycogen (insulin-signalled enhancement of glucose flux through the set of glycogenic enzymes), and facilitated glucose transport into adipose and muscle cells (insulin-signalled deployment of GLUT-4, a tissue-specific glucose transport protein). During starvation, glucose is released from the liver (glucagon-signalled enhancement of the flux through the steps of glycogenolysis and gluconeogenesis). Corresponding changes in the magnitude and
direction of fluxes of fatty acids and amino acids into and out of the blood are also observed. In effect, all cells of the body are guaranteed a dependable caloric environment regardless of metabolic demand and the starve-feed status. Unlike unicellular organisms that must adapt to a changing nutritional environment, tissue-bound animal cells respond to a pattern of extracellular signals which logically subserve the needs of the whole organism in achieving internal homeostasis.

Liver and adipose tissue are the major players in fatty acid and TAG synthesis. Glucose, amino acids and fatty acids all readily flow into the pathway of synthesis of TAG. Glucose and any amino acid that can be converted into acetyl CoA are incorporated into the formation of palmitic acid by cytosolic acetyl CoA carboxylase and the fatty acid synthase complex. Other long chain fatty acids are derived from palmitate by enzymes on the endoplasmic reticulum. These, supplemented with dietary fatty acids (including linoleic and arachadonic which are not synthesized in animals), form TAG by acylation of 3-phosphoglycerol, a byproduct of glycolysis. Within the lumen of the liver endoplasmic reticulum most of the TAG is incorporated into the structure of the “very low density lipoproteins” (VLDL) along with phosphatidyl choline and cholesterol [4,5]. This liver secretory product circulates through the capillary beds of many tissues and progressively donates its fatty acids to cells through the action of lipoprotein lipase for tissue fatty acid oxidation or the formation of storage TAG. Adipose TAG is derived from this source as well as by the abundant direct synthesis of fat from glucose (which provides the carbon for both fatty acids and glycerol).

Metabolic pathways can be thought of in terms of (a) the intrinsic activity constants of the individual enzymes that make up interconnected sequential multi-enzyme networks, and (b) the actual realized flux of organic metabolites on their way to complete oxidation or to synthesis of cell components, including stored metabolic fuels. The purpose of metabolic control in the final analysis is to dictate the magnitude and direction of the flux of organic intermediates. To this end, the activity parameters of enzymes are modulated, viz. the effective quantity of an
enzyme in a region, reflected in its $V_{MAX}$ value, and the binding affinities of its substrates and products, reflected (inversely) in its $K_m$ values.

The cell so defined awaits the input of accessible metabolites ("substrate pressure"). Since the latter are generally under homeostatic control (blood nutrient homeostasis), metabolic fluxes are diverted by extracellular signals that cause the quantities of certain enzymes to vary ($V_{MAX}$) through adjustments in their rates of enzyme synthesis (selective gene expression), and/or the tuning of $K_m$ values, principally by imposing a conformational change in the protein-enzyme structure (through covalent modulation and/or binding of positive or negative allosteric effectors). Not to be overlooked in this scheme are the minute quantities and diverse locations of intracellular coenzymes which shuttle between two extreme forms (e.g. NADPH/NADP; ATP/ADP). In theory, metabolic flux is in part, limited by the rates of these interconversions (e.g. ATP turnover coupled to metabolic demand and oxidative phosphorylation, NADPH generation coupled to lipogenic reductive synthesis).

Nutrient excess and deficiency are blunted by flux diversions into and out of the storage fuel mode. Thus in feeding the enzymes catalyzing glycogen and TAG synthesis are positively modulated and/or their net formation is enhanced. The same enzymes are usually down-regulated during starvation, while the activities of an opposing set of enzymes, catalyzing glycogen and TAG utilization, are enhanced.

COORDINATE INDUCTION/REPRESSION OF LIPOGENIC ENZYMES BY INSULIN/GLUCAGON

Proteins in cells of mature multicellular organisms are continuously synthesized at various rates, counterbalanced by first order degradation, so that characteristic (genetic) steady state concentrations of these proteins are maintained (Schoenheimer dynamic state) [6]. Exceptions to this pattern are seen in cells creating proteins for export, in cells that are actively dividing (e.g. during hematopoiesis and epithelial regeneration), and in cells responding to adaptive signals to synthesize (or not) a set of enzymes that permit a diversion of metabolic flux.
Selective net synthesis of a set of enzymes in liver and adipose tissue catalyzing the formation of fatty acids was recognized early as a response to feeding in animals. Presently an examination of the metabolic pathway leading from glucose through acetyl CoA to fatty acids and TAG reveals at least ten enzymes that are coordinately induced (increase in enzyme concentration and activity) coincident with an increase in the flux of glucose and acetyl CoA into fatty acids (rate of incorporation of isotopic precursors into fatty acids, in vitro and in vivo): glucokinase, glucose 6-phosphate dehydrogenase (NADPH), 6-phosphogluconate dehydrogenase (NADPH), pyruvate kinase, malate enzyme (NADPH), ATP-citrate lyase, acetyl CoA carboxylase, fatty acid synthase complex, δ9 acyl CoA desaturase and HMG CoA reductase (cholesterol synthesis) [7,8,9]. This list includes three enzymes that generate NADPH for reductive steps in fatty acid and cholesterol synthesis. Although cholesterol is not a stored metabolic fuel, key enzyme in the cholesterol synthesis pathway from acetyl CoA are similarly induced [10,11]. It is noteworthy that multiple enzymes share the flux control, suggesting that the increase in flux directed toward fatty acid synthesis can be accomplished without flooding the cell with high concentrations of pathway intermediates [7]. The rise in the level of the induced set of enzymes is self-limiting. As the mass of enzymes rise, the first order rate of degradation also increases [7,12].

This induction process which is coincident with the release of insulin during feeding is now understood in terms of insulin binding to its receptor on the plasma membrane of liver parenchymal cells and adipocytes. Excitation of the signal transduction pathway linked to the insulin receptor leads ultimately to the nucleus where insulin-specific transcription factors are activated and bind to the promoter regions of the lipogenic enzyme genes. The pyruvate kinase gene provides a well-studied representative of the lipogenic set [13,14]. Among members of the lipogenic gene set, homologous insulin responsive elements (DNA promoter regions, IREs) have been identified [13,14]. Corresponding elevations in specific mRNA concentrations have been measured as a prelude to the insulin-signalled rise in lipogenic enzyme activity [8,9].
During feeding another set of enzymes is repressed, e.g. phosphoenol pyruvate (PEP) carboxykinase, and glucose-6-phosphatase [13,14,15]. These are key enzymes in the gluconeogenic pathway. At the level of gene control insulin appears to repress the expression of this set of enzymes at the time when glucagon levels are low. At the other extreme, progressive starvation, the insulin/glucagon ratio is low permitting induction of the gluconeogenic set of enzymes while expression of lipogenic set is severely diminished.

Our earliest appreciation of the molecular mechanisms involved in metabolic signalling was the discovery of the “second messenger” cyclic AMP (cAMP) and cAMP-dependent protein kinase generated in response to epinephrine or glucagon [16,17], in the context of glycogenolysis. Induction of the gluconeogenic enzyme set (and repression of the lipogenic set) initiated by glucagon in liver is similarly transmitted through cAMP-protein kinase. The liberated catalytic subunit migrates to the nucleus to phosphorylate a key transcription factor (CREB) thereby permitting it to bind to CRE (DNA) promoter regions (cAMP Responsive Elements) of the gluconeogenic gene set. [13,14]. Although this remains research in progress, CRE regions in the lipogenic set of genes allow repression of their expression when the insulin-specific transcription factors are diminished.

**CONTROL OF LIPOGENESIS THROUGH REVERSIBLE PHOSPHORYLATION**

The elucidation of the signal system for control of glycogenesis and glycogenolysis ushered in the universe of metabolic regulation by reversible phosphorylation [16-19]. Its description at the molecular level continues to serve as a model for short-term control of lipogenesis.

It is stated that a third of cellular proteins are subject to phosphorylation by ATP (GTP). Phosphorylation of a protein/enzyme drives its conformation between two extremes, in the case of an enzyme between catalytically active and less active states. Not infrequently, the phosphorylation site resides in a regulatory protein subunit tightly apposed to a catalytic subunit whose activity is regulated by the degree of phosphorylation. Phosphorylation of specific amino acid side chains (serine, threonine or tyrosine) of protein is accomplished by
protein kinases through stereospecific binding. The added phosphate groups are removed by hydrolysis on binding with specific protein phosphatases. The phosphorylation state of a protein/enzyme may be considered as a dynamic balance between the activity of a specific protein kinase and protein phosphatase. The kinase and phosphatase are physically and functionally linked to other proteins that affect their catalytic activities. These include protein kinases and phosphatases that are often members of a signal transduction cascade linked to cell surface receptors through protein-protein binding [19, 20].

Three key enzymes in the synthesis and utilization of liver glycogen are controlled by their phosphorylation state [17-20]. All become dephosphorylated (by protein phosphatases) in response to feeding (high insulin/glucagon ratio), and all three are phosphorylated in response to starvation (low insulin/glucagon ratio). Glycogen synthase is inactive when phosphorylated by cAMP protein kinase, while phosphorylase kinase is active after phosphorylation. It then proceeds to phosphorylate and activate the enzyme phosphorylase (which catalyzes the phosphorolysis of glycogen). Thus glucagon in starvation leads to glycogenolysis via cAMP formation (adenylate cyclase activation by the tripartite GTP-binding protein adjacent to the glucagon receptor) and activation of the cAMP-dependent protein kinase. This facile system operates in starvation well before the decline in mass of lipogenic enzymes (glucagon-signalled repression of lipogenic gene set; see before).

During feeding when the insulin/glucagon ratio is elevated, the glycogen enzyme set in liver becomes dephosphorylated since cAMP protein kinase activity is diminished, and a glycogen-bound protein phosphatase-1 activity is enhanced [20,21,22]. Studies in skeletal muscle have revealed the possible signal transduction steps that lead from the insulin receptor to the activation of a protein phosphatase-1G (through its glycogen-bound regulatory subunit).

A similar linkage between insulin and activation of protein phosphatases also exists in liver. Glycogen synthesis is accelerated by glucose and its first intracellular metabolic product glucose-6-phosphate [18,22]. During feeding, dephosphorylation of phosphorylase-a is
enhanced in the presence of glucose. Also, glucose-6-phosphate (the product of increasingly active glucokinase) is a positive allosteric effector for glycogen synthase dephosphorylation [22]. This synergy is readily visualized with the recent finding of a liver protein bound to glycogen that may act as an organizing scaffold embracing glycogen synthase, phosphorylase kinase, phosphorylase and the catalytic subunit of protein phosphatase-1 [23]. Glucose also functions as a positive allosteric effector promoting the induction of acetyl CoA carboxylase in preadipocytes (see previous section) [24]. Glucose enhances the activity of the catalytic subunit of a protein phosphatase in the nucleus leading to the dephosphorylation of transcription factor, Sp1, thereby facilitating its binding to "glucose response elements" in the promoter II region of the acetyl CoA carboxylase gene.

The endocrine control of glycogen storage and utilization is emphasized to introduce mechanisms that may be linked to the large set of interconvertible enzymes catalyzing the synthesis of fatty acids from glucose and acetyl CoA, and the utilization of stored TAG through lipolysis to free fatty acids. In addition to the glycogen enzymes, this group includes 6-phosphofructo-2-kinase, pyruvate kinase, pyruvate dehydrogenase, acetyl CoA carboxylase and adipose hormone-sensitive lipase. All members of this group become dephosphorylated in response to insulin (feeding), and phosphorylated in response to glucagon (starvation) [25]. Thus the insulin-signalled dephosphorylated mode enhances glycogenesis and lipogenesis and impairs glycogenolysis, gluconeogenesis and lipolysis. The opposite pattern obtains in response to glucagon (the phosphorylated mode of the same enzyme set).

In 1973, two key lipogenic enzymes were found to be controlled through phosphorylation. Both acetyl CoA carboxylase (the major limiting enzyme in fatty acid synthesis) and HMG CoA reductase (cholesterol synthesis) were inactivated in vitro by phosphorylation, and restored to full activity by the action of protein phosphatases [26,27,28,29,30]. In 1975, Harris reported that dibutyl cAMP impaired fatty acid synthesis in rat hepatocytes (Arch. Biochem. Biophys., 169, 168-180), while Geelen found that insulin stimulated lipogenesis in hepatocyte cultures (FEBS Lett, 58, 334-339).
Studies with liver acetyl CoA carboxylase in vivo showed a correlation among several changes in property ten minutes after injection of glucagon: specific enzyme activity (diminished), phosphorylation (increased), sensitivity to the positive allosteric effector citrate (diminished), and the degree of oligomerization of enzyme subunits (diminished). The opposite pattern obtained after insulin injection [31]. In the long sequence of amino acids comprising liver acetyl CoA carboxylase, only three serines are found to be phosphorylated in vivo: 79, 1200 and 1215 [30,32]. In vitro, however, cAMP-dependent protein kinase phosphorylates serines 77 and 1200 (but not 79 and 1215), and the degree of inactivation of the enzyme is not impressive [30,32].

HMG CoA reductase is an integral homodimeric protein of the endoplasmic reticulum in liver and other tissues firmly seated in the membrane with eight transmembrane helices and connected to a long cytosolic carboxy terminal extension which harbors the catalytic site and a single phosphorylation site [33,34]. Early in vitro phosphorylation studies showed that the catalytic subunit of cAMP protein kinase had no effect on the activity of the native HMG CoA reductase [27]. However, a new protein kinase was partially purified from the cytosol of rat liver that severely diminished the catalytic capacity of microsomal HMG CoA reductase [26,27,35]. Ethanol-treated protein phosphatase reactivated the phosphorylated reductase. Interestingly, the same phosphatase preparation inactivated reductase kinase. Subsequently, a “reductase kinase kinase” was separated that restored reductase kinase activity. Thus, a bicyclic phosphorylation system was identified that controlled cholesterol synthesis through modulation of HMG CoA reductase, long considered the limiting enzyme in this lipogenic pathway [27,35]. Further examination of this system in Hegart’s laboratory showed that reductase kinase activity was stimulated by micromolar concentrations of 5'AMP [36].

Hardie and coworkers [30] purified a liver cytosolic protein kinase that caused total inactivation of acetyl CoA carboxylase. This preparation shared properties with HMG CoA reductase kinase including copurification and sensitivity to 5'AMP. The homogeneous enzyme (63 kDa) was now designated 5'AMP-dependent protein kinase (AMPK) and was recognized
as an essential modality in the coordinate short-term control of both acetyl CoA carboxylase and HMG CoA reductase. Kim and coworkers introduced combinations of specific mutations of the phosphorylation sites in the cDNA of acetyl CoA carboxylase, and recovered the altered forms of the enzyme from the transfected cells for in vitro protein kinase assays. It was concluded that serine 79 was the principal site for 51 AMP protein kinase attack and serine-1200 for cyclic AMP protein kinase [32]. Both sites are phosphorylated in hepatocytes after glucagon treatment [30].

With a synthetic peptide substrate virtually identical to the sequence on either side of serine-79 (SAMS peptide) a convenient in vitro assay for AMP-protein kinase [37] permitted the further purification of both the kinase and the AMP-protein-kinase-kinase [38,39]. Highly purified AMP protein kinase from rat liver consists of three subunits α, the catalytic subunit, (63 kDa), β (38 kDa) and δ (35 kDa) which probably function as a compact heterotrimeric complex [40]. The catalytic subunit of the purified kinase-kinase (58kDa) is also accompanied with “subunits” (total native molecular mass 195kDa) [39]. Threonine 172 of the catalytic subunit of AMP protein kinase is phosphorylated by the kinase kinase. The activity of kinase kinase is not affected by protein phosphatase [39].

With the purified bicyclic protein kinases, 5′AMP is now known to influence not only the activity of the kinase directly, but also to enhance the phosphorylation catalyzed by the kinase-kinase, and to impair the inactivation of AMP kinase by added phosphatases [30,39]. Interestingly, long-chain acyl CoA, the classic negative feedback signal of fatty acid synthesis during starvation [3,7], stimulated the activity of AMP protein kinase kinase in vitro [30].

Incubation of hepatocytes with fructose or under anoxic conditions increases the intracellular levels of 5′AMP at the expense of ATP. Under these conditions, both fatty acid and cholesterol synthesis are severely diminished [30,41], an effect directly attributed to stimulation of AMP-sensitive protein kinase. A synthetic analog of AMP (5-amino 4 imidazole carboxamide ribotide, “AICAR”) stimulates AMP protein kinase 10-fold in vitro [42].
corresponding nucleoside is taken up by rat hepatocytes and converted primarily to the monophosphate thereby causing a parallel inhibition of fatty acid and cholesterol synthesis—a finding of potential clinical importance [42].

It is doubtful if 5'AMP is an intracellular signal or effector of lipogenesis under ordinary circumstances. For example, the level of AMP does not undergo a diurnal variation [30]. The question is raised whether or not insulin and glucagon short-term signalling influences the activity of AMP protein kinase. The first study with hepatocytes (1979) indicated that insulin enhanced HMG CoA reductase expressed activity (KF present) at the same time that (then designated) reductase kinase activity was depressed. The opposite pattern obtained with glucagon [43]. Witters laboratory in 1992 [44] observed in hepatoma cells that insulin caused AMP protein kinase inhibition prior to activation of acetyl CoA carboxylase, both activities requiring the presence of protein phosphatase inhibitors during the preparation for assay. The differential change in activity of AMP kinase with time due to insulin was not affected by AMP. Munday, et al., reported studies carried out in rats that were starved over a period of 48 hours then refed up to 24 hours [45]. Six hours into starvation expressed liver acetyl CoA carboxylase activity was depressed, Ka for citrate activation was increased, while AMP protein kinase activity rose (as did cAMP-dependent protein kinase). Insulin levels fell significantly. At 6 hours total acetyl CoA carboxylase (enzyme mass) was not changed. This total activity reached a minimum at 48 hours starvation (repression of enzyme synthesis). On refeeding the parameters cited above reversed in value over a period of 6 hours refeeding. As insulin levels rose AMP-protein kinase activity fell (as did cAMP-dependent protein kinase). Expressed acetyl CoA carboxylase rose, with total activity following slowly. This kind of in vivo analysis demonstrated that the state of phosphorylation of AMP-dependent protein kinase does respond to insulin and glucagon in liver.

The signal transduction pathways leading from insulin and glucagon receptors to AMP-dependent protein kinase are not understood. One option is to think in terms of protein phosphatases as logical targets. While protein phosphatase-1 plays significant regulatory roles
in controlling glycogen synthesis (see before) the present evidence points to protein phosphatase 2A acting on liver phosphorylated acetyl CoA carboxylase and HMG CoA reductase, while (okadaic acid-insensitive) protein phosphatase 2C removes phosphate from AMP-dependent protein kinase [30]. Phosphatase 2A is endowed with many possible regulatory subunit combinations, but phosphatase 2C is not [21]. Cyclic AMP-dependent protein kinase remains the primary intracellular signal linked to the glucagon receptor (see before). However, it does not phosphorylate AMP-dependent kinase. Signal pathways linked to the insulin receptor are certainly more elaborate (MAP kinase pathway reviewed in [20,21]). The involvement of the MAP kinase pathway in controlling glycogen-bound phosphatase 1G is in doubt. Also, other insulin-targeted modulations do not seem to depend on the MAP kinase sequence, e.g. [46]. The nature of insulin signal itself remains an area of active research, viz. the insulin-generated mediator that elicits several significant insulin responses [47].

SELF CONTROL

In the final analysis, the control of lipogenic metabolic flux (net formation of storage TAG) is designed to handle “substrate pressure”, an abundance of synthetic precursors. Our appetite for food may have the final dictate in control, balanced by the genetic capacity of adipose tissue for oxidative metabolism uncoupled from metabolic demand. A new appreciation of endocrine controls that influence appetite in experimental animals is rapidly emerging. Among the latest satiety factors is leptin, secreted by adipocytes, which ultimately reaches receptors in regions of the hypothalamus long known to be important in appetite modulation [48]. Leptin, the protein product of the ob gene, impairs the appetite-stimulating response of neuropeptide Y produced in the hypothalamus [49, 50]. For good measure ob gene expression in preadipocyte cultures suppresses the induction of acetyl CoA carboxylase engendered by lipogenic hormones [51]. The human adipose agouti gene product stimulates appetite by impairing the binding of melanocortin (aMSH) to its receptor in the hypothalamus which normally diminishes the appetite enhancing effect of neuropeptide Y [52]. Another hypothalamic hormone, glucagon-like peptide-1, powerfully inhibits feeding in rats [53].
Studies along another direction indicate that expanding TAG reserves in adipose tissue may limit TAG accumulation through autocrine feedback signalling, supporting the concept of genetic set points for energy balance and weight regulation. A close (59%) homolog of the uncoupling protein of brown fat mitochondria (UCP-1 gene product) has been identified in white adipose tissue (UCP-2) [54]. This protein pumps fatty acid anions from the mitochondrial matrix space thereby providing a futile cycle of protons and uncoupled oxidative metabolism. UCP-2 is upregulated by fat feeding. Tumor necrosis factor (TNFα), an autocrine product of adipose tissue, blocks the adipocyte insulin receptor response thereby diminishing GLUT-4 deployment (glucose uptake) and permitting lipolysis of TAG. Human obesity is often accompanied by insulin resistance (non-insulin-dependent diabetes mellitus) [55].
REFERENCES


ANTIBIOTIC RESISTANCE: A WORLDWIDE PROBLEM

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ABSTRACT

Since their discovery, antibiotics have proved to be effective for the control of many bacterial infectious diseases. However, it was soon evident that bacteria rapidly became resistant to antibiotics. For the first time since antibiotics were introduced about 50 years ago, antibiotic resistance has become a global problem of vast scope and complexity.

The variety of infections that demonstrate antibiotic resistance is striking, ranging from community-acquired pathogens such as Shigella spp., and Haemophilus influenzae to hospital-acquired infections due to Staphylococcus aureus and Enterobacter spp. The problem has appeared worldwide, both in developed and developing nations.

Emergence of clinically important antibiotic resistance

Community-acquired pathogens:

Among the community-acquired pathogens, enteric pathogens were the first to become resistant to antibiotics. In 1970s Shigella spp., and Salmonella spp. became resistant to commonly used antibiotics, such as ampicillin, chloramphenicol and tetracyclines, first in developing countries, then in developed ones. Presently antibiotic resistance is common in these agents and in some cases it is quite difficult to find an effective antibiotic to treat infections caused by these pathogens, especially in children. Multi-drug resistant Salmonella typhi is also a major problem in developing countries. In 1980s penicillin-resistant N. gonorrhoeae was reported. In recent years resistance to quinolones in this organism was found. Ampicillin-resistant Haemophilus influenzae is another community-acquired pathogen, worldwide 20% to 40% are resistant to ampicillin and amoxicillin. In late 1980s and early 1990s Streptococcus pneumoniae, a common respiratory pathogen became the topic of discussions. This organism was sensitive to penicillin, however in recent year there are reports
of resistance to penicillin in many regions of the world. In some countries (such as Hungary and Spain) penicillin-resistance in pneumococcus is up to 35% to 40%. Some of these organisms are also resistant to other Beta-lactam antibiotics.

Hospital-acquired pathogens:

In 1960s, shortly after the use of methicillin and similar agents for staphylococcal infections, resistance to this group of antibiotics was reported. Presently both *Staphylococcus aureus* and coagulase-negative staphylococcus are not only resistant to methicillin but also to aminoglycosides, clindamycin, macrolides and quinolones. Gram-negative bacilli, most common organisms causing nosocomial infections in many hospitals, are resistant to many antibiotics. Among them, multi-drug resistant Klebsiella spp., Enterobacter spp., and Pseudomonas spp. are the most important ones. In recent years Enterococcus became a major problem in many teaching hospitals in USA. Vancomycin resistant enterococcus is a major pathogen in intensive care unit nosocomial infections. Another nosocomial pathogen is multi-drug resistant *Mycobacterium tuberculosis*. It is also an important health hazard for health care workers taking care of HIV infected patients.

Mechanisms of resistance:

The antimicrobial agents in clinical use were developed to inhibit targets unique to prokaryotic cells. Some examples include the bacterial cell wall, the bacterial ribosome, and bacterial DNA gyrase. Resistance reflects the ability of a microorganism to avoid the inhibitory or lethal activity of an antimicrobial agent. "Intrinsic resistance" to an antimicrobial agent characterizes resistance that is an inherent attribute of a particular species; these organisms may lack the appropriate drug-susceptible target or possess natural barriers that prevent the agent from reaching the target. "Circumstantial resistance" is the difference between the in vitro and in vivo effects of an antimicrobial agent. Agents that appear to be active in the laboratory may be ineffective in vivo because of failure to reach a site of infection. "Acquired resistance" reflects a true change in genetic composition of a bacterium so that a drug that once was effective in vivo no longer is active.
The major resistance mechanisms are shown on Table 1. These include limiting the intracellular concentration of the antibiotic, neutralization of the antibiotic by enzymes, alteration of the target, and elimination of the target. Bacteria may employ or combine multiple mechanisms against a single agent or class of agents. A single change may result in development of resistance to several different antibiotics.

<table>
<thead>
<tr>
<th>Table 1: Resistance mechanisms to antimicrobial agents</th>
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<tr>
<td>1. Diminished intracellular drug concentration</td>
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<tr>
<td>a. Increased efflux</td>
</tr>
<tr>
<td>Tetracycline, quinolones</td>
</tr>
<tr>
<td>b. Decreased outer membrane permeability</td>
</tr>
<tr>
<td>Beta-lactams, quinolones</td>
</tr>
<tr>
<td>c. Decreased cytoplasmic membrane transport</td>
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<tr>
<td>Aminoglycosides</td>
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<tr>
<td>2. Drug inactivation (inactivating enzymes)</td>
</tr>
<tr>
<td>Beta-lactams, aminoglycosides, chloramphenicol</td>
</tr>
<tr>
<td>3. Target modification</td>
</tr>
<tr>
<td>Quinolones (gyrase modifications), rifampin (DNA polymerase binding), Beta-lactams (PBP changes), macrolides (rRNA methylation)</td>
</tr>
<tr>
<td>4. Target bypass</td>
</tr>
<tr>
<td>Glycopeptides (vanA, vanB), trimethprin (thymine-deficient strains)</td>
</tr>
</tbody>
</table>

The consequences of antibiotic resistance:

Medical consequences:

The emergence of antibiotic-resistant isolates has enormous clinical implications. Morbidity, mortality, and costs associated with disease increase when the pathogen is antibiotic resistant. Morbidity and mortality rates increase because of the delayed initiation of effective therapy for a specific infection, particularly when resistance emerges to the drug of choice or to standard empiric therapy.
The use of new antibiotics to overcome the resistance problem may carry increased medical cost, and additional new adverse effects. Antibiotic resistance can lead to an increase in the incidence of disease. The most scared consequence of resistance is that some infections may become untreatable. This has already happened with some isolates of enterococci and tuberculosis.

**Economic consequences:**

Overlying the intangible costs of increased disease prevalence, morbidity, and mortality are the quantifiable monetary costs of antibiotic resistance. Individuals and society must pay for the higher cost of the newer antibiotics needed to combat resistant bacteria.

The problem of the higher cost of additional drug therapy is exacerbated by the financial burden of prolonged hospitalization. The likelihood of hospitalization and the length of hospital stay are typically at least twice as much for patients infected with antibiotic-resistant bacterial isolate compared with patients infected with antibiotic-sensitive one.

It is costly, time-consuming, and complex to treat a hospitalized patient who has an antibiotic-resistant infection and to control the spread of resistant isolates. Based on developed models, the annual cost of antibiotic resistance in the United States is estimated to be $35 billion.

**What to do?**

The most important factor that cause development of antibiotic resistance is the misuse of antibiotics. The misuse accelerates the resistance process, and it is appropriate to focus on misuse because it is, by definition, preventable.

Antibiotic misuse takes many forms. The use of antibiotics in clinical medicine and veterinary medicine has led to major changes in the genotypes and phenotypes of almost all pathogenic bacteria.
In many parts of the world, including Turkey, antibiotics can be purchased by the general public directly from pharmacies, without the need of a physician’s prescription. Free access to antibiotics by people who are not trained in their appropriate use is an open invitation to overuse and misuse.

A too-short or inadequate course of therapy provides an excellent breeding ground for resistance since the infection will not be eradicated. The strains that are resistant to the antibiotic will be selected and they will become the dominant pathogens. Inadequate or misdirected antibiotic therapy often occurs when the goal is prophylaxis.

Patients often stop their antibiotic treatment prematurely, believing that if they feel better, it is not necessary to take the full course. This is an excellent way to select for antibiotic resistance, the situation is exacerbated when patients stockpile the remaining pills for self-medication at a later date.

Health care professionals also bear responsibility for fostering antibiotic resistance. Physicians must know when to prescribe a specific antibiotic and for which bacteria. Empiric therapy should be initiated only when justified by the likely presence of bacterial infection.

The increased use of invasive procedures, the AIDS epidemic, the increased number of immunocompromised patients, the crowding and poor sanitation in urban ghettos, and the mobility of people worldwide all contribute to the acquisition and spread of infections caused by resistant organisms.

With all these data it is clear that appropriate use of antimicrobial agents is the key to prevent development of antibiotic resistance.
REFERENCES


VANADIUM: A POTENTIAL THERAPEUTIC AGENT FOR DIABETES
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ABSTRACT

We demonstrated in 1985 that vanadium administered in the drinking water to streptozotocin (STZ) diabetic rats restored elevated blood glucose to normal. Subsequent studies have shown that vanadyl sulfate can lower elevated blood glucose, cholesterol and triglycerides in a variety of diabetic models including the STZ diabetic rat, the Zucker fatty rat and the Zucker diabetic fatty rat. Long-term studies of up to one year did not show toxicity in control or STZ rats administered vanadyl sulfate in doses that lowered elevated blood glucose. In the BB diabetic rat, a model of insulin-dependent diabetes, vanadyl sulfate lowered the insulin requirement by up to 75%. Vanadyl sulfate is effective orally when administered by either single dose or chronic doses. It is also effective by the intraperitoneal route. We have also been able to demonstrate marked long-term effects of vanadyl sulfate in diabetic animals following treatment and withdrawal of vanadyl sulfate. Because vanadyl sulfate is not well absorbed we have synthesized and tested a number of organic vanadium compounds. One of these, bis(maltolato)oxovanadium(IV) (BMOV), has shown promise as a therapeutic agent. BMOV is 2-3x more potent than vanadyl sulfate and has shown less toxicity. The mechanism of action of vanadium is currently under investigation. Several studies indicate that vanadium is a phosphatase inhibitor and vanadium can activate serine/threonine kinases distal to the insulin receptor presumably by preventing dephosphorylation due to inhibition of phosphatases. Short-term clinical trials using inorganic vanadium compounds in diabetic patients have been promising.

Keywords: vanadium, insulin resistance, diabetes, hypertension, BMOV
INTRODUCTION

More has been learned about diabetes in the last two decades than in any previous time and our understanding of the complex inter-relationship between insulin action, insulin resistance, lipid and carbohydrate metabolism has been greatly enhanced. One observation has been the intriguing discovery that the element vanadium, can mimic and enhance the actions of the peptide insulin. Since the first demonstration of the in vivo insulin-mimetic effects of vanadium in experimental models of diabetes [1] to the current data demonstrating its benefits in human diabetes comes the compelling question: will vanadium be a useful substitute for insulin? Some of the key aspects of vanadium biology in relation to the glucose-insulin cycle that exemplify the insulin-mimetic, antihyperglycemic and antihypertensive effects of this unique trace element are reviewed below.

BACKGROUND

Vanadium was discovered in 1813 by del Rio, who gave it the name panchromium because of its color changes when passing through various oxidation states and rediscovered in 1831 by the Swedish chemist Nils Gabriel Sefstrom, who named the compound Vanadis, a nickname of the Germanic goddess of beauty. In humans, the total body pool of vanadium is estimated to be around 100-200 ug [2]. Vanadium exists in several valence states (-3, -1, 0, +1 to +5) and the expression of a given form is highly pH dependent. In biological systems vanadium is found predominantly as vanadate (+5) and vanadyl (+4) forms. In the plasma, vanadium exists in both oxidation states. Approximately 90% is bound to proteins (predominantly transferrin)[3]. Most ingested vanadium is transformed in the stomach to VO\(^{1+}\) and remains in this form as it passes through the duodenum. Vanadium is preferentially distributed in the bone, kidney and liver following i.p. injection; the bone representing the main storage depot for vanadium [4].
IN VITRO INSULIN-MIMETIC ACTIONS OF VANADIUM

Vanadium exerts insulin-like actions on both carbohydrate and lipid metabolism including glucose transport, glucose transporter translocation, glycolysis and glycolytic enzymes, glucose oxidation, glucose output, glycogen synthesis and lipolysis [5-16].

IN VIVO INSULIN MIMETIC ACTIONS OF VANADIUM

Heyliger et al. [1] were the first report to describe the in vivo insulin-mimetic glucose lowering actions of vanadium in experimental diabetes. In this study, chronic treatment of streptozotocin (STZ) diabetic rats with sodium orthovanadate normalized hyperglycemia and improve the depressed cardiac function but did not increase plasma insulin levels. The ability of vanadium to cause euglycemia without increases in plasma insulin levels indicates an improvement in insulin sensitivity. Recently we demonstrated that vanadium decreases insulin resistance when assessed using the euglycemic hyperinsulinemic clamp [17]. In 1987, Meyerovitch et al demonstrated that chronic sodium metavanadate administration also lowered plasma glucose levels and enhanced glucose transport in both liver and muscle [18]. A dose-response relationship between vanadate and its glucose lowering effects was described by Brichard et al. in 1988 [19]. Some in vivo effects of vanadium administration in STZ-diabetic rats are listed in Table 1.

Table-1 In Vivo Effects of Vanadium in STZ-Diabetes

- Amelioration of insulin resistance reflected by a greater glucose-lowering effect of vanadium-treated rats to insulin (20)
- Normalization of both basal and stimulated hepatic glucose production by chronic vanadium administration (21)
- Enhanced insulin sensitivity in vanadium treated rats correlates with restoration of insulin stimulated MAP and S6 kinase activities in skeletal muscle (23)
Chronic vanadium treatment corrects abnormalities in glycolytic enzymes i.e. phosphofructokinase-2 and glucokinase (24).
Restoration of glycogen synthase and phosphorylase activities (25).
Aberrations in the tissue-specific expression of 2 isoforms of glucose transporter in STZ-diabetes are normalized by vanadium treatment (26).
Amelioration of oxidative stress (27).
Long-term effects on glucose metabolism following oral treatment and withdrawal (31).
Prevention of secondary cardiac complications (1,30).

Following our demonstration of the in vivo antihyperglycemic effects of vanadate, work in our laboratory and that by others revealed that high concentrations of vanadate used in the drinking water was accompanied by adverse effects including diarrhea and death. Subsequently we have learned that the deaths found in these studies are due to dehydration because the animals do not drink. If diabetic rats given vanadium are kept properly hydrated the deaths do not occur. At the time however we were looking for a less toxic compound. Based on observations (based on the LD₅₀) that sodium orthovanadate was 6-10 times more lethal than vanadyl sulfate [28-29], we hypothesized that the vanadyl rather than the vanadate form may be more appropriate for in vivo administration. We have since shown that STZ-diabetic rats given vanadyl sulfate exhibit normal plasma levels of glucose, lipids, creatinine and thyroid hormone levels [32]. In addition, abnormalities in isolated working heart function and glycerol output from adipose tissue of diabetic animals are also corrected in such animals. These results established the effectiveness and relative safety of vanadyl sulfate in treating experimental diabetes.

An unusual and interesting observation found with vanadyl sulfate treatment was made in the study by Ramanadham et al. Demonstrating the sustained prevention of myocardial and metabolic aberrations in diabetic rats following withdrawal from vanadyl sulfate treatment [30]. It was found that after 3 weeks of oral treatment with vanadyl sulfate followed by 13 weeks of no treatment, plasma concentrations of glucose insulin, lipids and thyroid hormones in the
STZ-treated animals remained at control levels. Secondary complications such as cataracts and myocardial dysfunction also did not occur in these animals compared with untreated diabetic rats. These findings revealed for the first time the long-term effectiveness of short term treatment of diabetic rats with oral vanadyl sulfate.

To examine whether the anti-diabetic effects of vanadyl sulfate could be shown after the diabetic state was well established, Cam et al. Examined the effectiveness of vanadyl sulfate when given up to 17 days after induction of diabetes [31]. Vanadyl sulfate was administered in the drinking water (0.75 mg/ml) from 3, 10 and 17 days after STZ injection and treatment was then maintained for 5 months. Glucose tolerance and adipose tissue function were normalized in vanadyl treated diabetic rats in all treated groups thus supporting the concept that the efficacy of vanadyl sulfate as an insulin-mimetic is not secondary to protection of the pancreatic β cells from the cytotoxic effects of STZ by vanadium.

The concentration-dependent effects of oral vanadyl treatment and the in vivo interaction of vanadyl with insulin was examined by Ramanadham et al. [20]. Vanadyl sulfate treatment in the spontaneous BB rat (a model of absolute insulin deficiency) reduced by 75% the exogenous insulin required to prevent glycosuria in BB diabetic rats. This study established that at least some insulin is required for vanadium to produce its effects in vivo. The beneficial effects of vanadium have also been demonstrated in other models of Type I and Type II diabetes (Table 2).

<table>
<thead>
<tr>
<th>Table 2 In Vivo Effects of Vanadium in Other Models of Diabetes</th>
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<tbody>
<tr>
<td><strong>Spontaneously Diabetic BB Rat</strong></td>
</tr>
<tr>
<td>Reduces the dose of insulin required to maintain glycosuria</td>
</tr>
<tr>
<td>(20)</td>
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<tr>
<td><strong>Partially Pancreatectomized Rats</strong></td>
</tr>
<tr>
<td>Improves insulin-sensitivity towards peripheral glucose uptake</td>
</tr>
<tr>
<td>in 90% pancreatectomized rats predominantly through correction</td>
</tr>
<tr>
<td>of muscle glycogen synthesis (33)</td>
</tr>
</tbody>
</table>
• Neonatal STZ-Diabetic Rats
  Vanadium treatment corrects basal and stimulated hepatic glucose production and peripheral glucose utilization (34)

• Genetically Obese fa/fa (Zucker) Rats
  Attenuates hyperinsulinemia and impaired glucose tolerance (35)

• Obese ob/ob Mice
  Attenuates hyperglycemia, improves glucose tolerance and hepatic glycogen content. Prevents pancreatic exhaustion of insulin (36).

• Zucker Diabetic Fatty Rats
  Attenuates hyperglycemia, hyperinsulinemia and hyperlipidemia. Restores glucose tolerance. (Yuen and McNeill, unpublished observations)

Both vanadate and vanadyl salts are poorly absorbed from the gastrointestinal (GI) tract and GI toxicity, notably diarrhea, has been reported. Our laboratory and others have synthesized a number of organic vanadium compounds with the aim of producing compounds that are safer, better absorbed and more potent.

Bis(maltolato)oxovanadium(VI)(BMOV), a maltol/vanadyl compound, was developed in collaboration with Dr. C. Orvig in the Department of Chemistry at the University of British Columbia, Vancouver, Canada [37]. BMOV is a successful example of a series of compounds designed specifically to be better absorbed orally by passive diffusion as a result of their properties of water solubility, electrical neutrality and low molecular weight [10,40]. BMOV was compared to vanadyl sulfate when administered acutely both by oral gavage and by intraperitoneal (i.p.) injection [38]. At the highest doses administered BMOV produced euglycemia in 100% of animals treated as compared to 80-90% with vanadyl sulfate. The oral ED_{50} test showed that BMOV was twice as potent as vanadyl sulfate (ED_{50}: 0.5 mmol/kg for BMOV vs. 0.92 mmol/kg for vanadyl sulfate) and 3 times as potent by i.p. injection.

In a chronic study, BMOV (0.75 mg/ml) was administered to STZ-diabetic rats in drinking water for 6 months. Plasma glucose returned to normal in 8/12 animals and heart function was
restored in all diabetic treated rats [38]. BMOV had no effect on body weight gain in control rats during the initial 10-week treatment period whereas vanadyl sulfate decreased body weight gain. Both vanadium compounds decreased plasma insulin levels in control-treated animals indicating an increase in insulin sensitivity [38]. Concern has been expressed in the literature about the toxicity of vanadium. In our laboratory, Dai et al. Have evaluated the effects of long-term vanadyl sulfate and BMOV treatment on several pathological determinants of STZ-induced diabetes [39,39a]. Chronic BMOV treatment completely prevented elevations in plasma urea, creatinine, alanine aminotransferase (ALT) and improved histological abnormalities in the kidney and liver from STZ-diabetic rats. No significant vanadium-induced toxicity was seen in these studies which used doses of vanadium that produced euglycemia in the STZ rats.

In fa/fa (fatty) Zucker rats, a model of non-insulin dependent diabetes BMOV reduced the markedly elevated plasma insulin levels. At these concentrations BMOV did not affect body weight gain in lean controls but did significantly reduce body weight in the fatty treated group. At a lower concentration BMOV did not affect food and fluid intake, body weight gain or plasma cholesterol levels in fatty treated animals but did significantly reduce plasma glucose, insulin and triglyceride levels. Oral glucose tolerance was also improved in the fatty animals by BMOV treatment [41].

Considerable epidemiological, clinical and experimental data support the association between essential hypertension and abnormalities in carbohydrate and lipid metabolism [42-44]. Insulin resistance and hyperinsulinemia appear to be major links between metabolic abnormalities and hypertension and are associated with a highly atherogenic risk profile, dyslipidemia and atherosclerosis. Logically then, drug interventions that improve these defects may also decrease blood pressure if this linkage occurs. We have employed vanadium (both vanadyl sulfate and BMOV) to examine the relationship between hyperinsulinemia, insulin resistance and hypertension [17,45-47] in both a genetic and an acquired model of hypertension, the spontaneously hypertensive rat (SHR) and the fructose-hypertensive rat. Vanadium
compounds reduced both plasma insulin and blood pressure in both animal models studied and the vanadium effect on blood pressure was reversed by administering insulin to restore plasma insulin levels to those of the untreated state. These data support the hyperinsulinemia: insulin resistance-hypertension hypothesis and may suggest a unique method of treating clinical hypertension. The mechanism(s) of the antidiabetic effects of vanadium in vivo are currently being investigated. Vanadium does affect various aspects of the insulin signaling pathway in vitro (Table 3) but the exact in vivo mechanism(s) at the cellular levels is not known.

Table 3 Suggested Effects of Vanadium on Insulin-Signalling Pathways In Vitro

- Stimulates Autophosphorylation of Insulin Receptors
- Increases Insulin Receptor Tyrosine Kinase Activity
- Stimulates Down-Regulation of Insulin Receptors
- Increases Insulin Receptor Binding
- Increases Protein Tyrosine Kinase Activity
- Increases Ser/Thr Protein Kinase Activity
- Inhibits Phosphotyrosine Phosphatase Activity

Vanadium may behave as a phosphate analog and stimulate protein-tyrosine phosphorylation by virtue of its inhibitory actions on phosphatases (PTPase) [48,49]. Early studies suggested that vanadium activated autophosphorylation of solubilized insulin receptors (IR) but not serine or threonine residues of the receptors, in a fashion analogous to insulin [9,50,51]. Vanadium was also shown to stimulate tyrosine kinase activity of the IR β subunit [50,52]. More recent work indicates a more downstream locus of action of vanadium in the insulin signaling pathway since vanadium was equally effective in stimulating glucose metabolism in rat fat cells when half the IRs had been inactivated by insulin overstimulation [53]. In addition, oral vanadium treatment failed to change IR kinase activity while exerting glucose lowering effects [54]. Recent evidence suggests that vanadium's effects are mediated via some post-
The long-term effects of vanadium treatment have been further investigated in our laboratory. Some diabetic animals appear to maintain a chronic euglycemic state following vanadium...
treatment withdrawal with only minor improvements in pancreatic secretory function. The treated rats apparently sustain an increased sensitivity to circulating insulin after vanadium treatment is withdrawn. Alternatively, tissue vanadium stores could be released and continue to exert antihyperglycemic effects but this appears to be highly unlikely. Cam et al. [59] recently addressed this issue and suggest that vanadium-induced amelioration of the diabetic state is related in part to the preservation of a functional portion of the pancreatic β-cells which initially survived STZ toxicity. This partial preservation of β-cells, although small in proportion to the total insulin store is critical for a chronic reversal of the diabetic state. Since plasma insulin values are still not fully restored other factors such as a long-term effect on the enhancement of insulin’s actions must also be operative [59].

Of great interest are the recent publications on the effect of vanadium administration on diabetic humans. Goldfine et al. Examined the effects of 2-week sodium orthovanadate administration in both Type I and Type II diabetic patients [60]. Treatment with sodium orthovanadate (125 mg daily in divided doses) lowered insulin requirements but had no effect on basal or C-peptide levels indicating no effect on insulin release. Two of the 5 insulin-dependent diabetic patients showed improvements in glucose utilization. More dramatic improvements were observed in Type II diabetic patients who displayed improved insulin sensitivity related to an enhancement of non-oxidative glucose disposal rates. Vanadium treatment did not affect hepatic glucose production in this study. Furthermore, basal MAP and S6 kinases were significantly activated in monocytes. The main side effect observed was diarrhea.

The effects of vanadyl sulfate (100 mg/day) for 3 weeks in six non-insulin dependent diabetic subjects has also been reported [61]. Treatment resulted in reduced fasting plasma glucose and HbA1c and no effect on plasma insulin levels. The observation that the beneficial effects on insulin-sensitivity persisted for up to 2 weeks following cessation of treatment; the latter observation is consistent with experimental studies described earlier. Two more recent reports
[62,63] have also shown positive effects of vanadyl and vanadate in non-insulin dependent patients.

Vanadium compounds have been shown to normalize the hyperphagia associated with experimental diabetes. In 1994, Malabu et al. claimed that the decreases in plasma glucose levels observed after administration of vanadate were entirely attributable to a reduction in food intake [64]. Yuen et al. In our laboratory recently conducted a detailed study to ascertain the effects of vanadium and of food restriction on plasma glucose levels and lipid in STZ-diabetic rats [65]. BMOV was administered daily in drinking water to STZ-diabetic rats for 6 weeks. Pair-fed groups were fed based on the intake for their respective counterparts from the previous day. BMOV reduced plasma glucose, triglyceride and cholesterol levels in diabetic rats without affecting plasma insulin levels whereas pairfeeding did not. BMOV, but not pair feeding, prevented the decreased cardiac function observed in STZ-diabetic rats. These data clearly indicate that the effects of vanadium are independent of any effect on dietary restriction. A possible problem in the study by Malabu et al. was that they provided the food to the pair-fed group once a day. Our observation is that the diabetic animals consume this small amount of food in a very short time. Thus the animals were fasted for a long time since blood was not drawn until the morning. This factor is crucial, since the reduction in plasma glucose levels in their study for the pair-fed diabetic groups was similar what we observed after prolonged (20-hour) periods of fasting.

Since the initial demonstration of the anti-diabetic effects of vanadium in vivo in 1985, significant advances have been made in understanding the glucose lowering properties and the mechanism(s) of action of vanadium. The exact cellular mechanism(s) and/or mediators involved in vanadium's action remain unknown but the action of vanadium may be mediated by a synergy between several post-receptor events in the insulin-signaling cascade. The development of various organic ligands showing improved absorption, potency tissue uptake and having decreased toxicity is important. BMOV exemplifies one such organically chelated complex that appears to be a potent insulin-mimetic and insulin-enhancer. BMOV has less GI
side effects and does not affect body weight gain and food and fluid intake in control-treated rats. Vanadium research demonstrating the antihypertensive effects of vanadium compounds in hyperinsulinemic and insulin resistant models of hypertension may also prove to be important. Early trials with vanadium in diabetic human volunteers have shown promising results consistent with experimental studies. Within the next few years the possible therapeutic roles of vanadium should be more clearly established.

ACKNOWLEDGEMENTS

Studies quoted in this paper from our laboratory have been supported by the Canadian Diabetes Association, the Medical Research Council of Canada, the Heart and Stroke Foundation of B.C. and Yukon and the Natural Sciences and Engineering Research Council of Canada. The administrative and technical assistance of Mary Battell is greatly appreciated. We thank Sylvia Chan for expert secretarial assistance.
REFERENCES


Green A. The insulin like effect of sodium vanadate on adipocyte transport is mediated at a post-insulin receptor level. *Biochem. J.* 238:663-669, 1993


MOLECULAR MECHANISMS OF INSULIN RESISTANCE. STRUCTURE AND SYNTHESIS OF A NOVEL INOSITOL-GLYCAN PSEUDO-DISACCHARIDE FROM BEEF LIVER WITH INSULIN-LIKE BIOACTIVITY IN VITRO AND IN VIVO.

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ABSTRACT

We isolated a putative insulin mediator or mimetic from beef liver by methods scaled up from rat liver where we had identified D-chiro-inositol and galactosamine as components. Structure was determined by degradative chemistry and 1 and 2 D NMR as a pseudo-disaccharide of pinitol (3-0-methyl D-chiro-inositol) and galactosamine chelated to a metal likely manganese. When infused, it effectively decreases hyperglycemia in diabetic rats to euglycemia in 60 min equally to a comparable dose of insulin without producing hypoglycemia. Two members of a family of Mg++ requiring phosphatases PDH and 2C are activated by left shifting the Mg++ dose response. Thus, two rate-limiting enzymes of non-oxidative and oxidative glucose disposal are activated by dephosphorylation, glycogen synthase and PDH. A decrease of chiro-inositol in urine and tissues in type II diabetic subjects and 1st degree relatives as well as in monkeys correlates with the degree of insulin resistance.

KEY WORDS: Insulin, Mediator, Inositol Glycan, Glycogen Synthase, Pyruvate Dehydrogenase.
INTRODUCTION

For greater than 45 years, I have had an interest in carbohydrate metabolism. With Carl and Gerty Cori, we originally established the multibranched structure of glycogen by serial enzymatic degradation with phosphorylase and debranching enzyme amylo-1,6-glucosidase [1]. Following the original discovery of glycogen synthase by Leloir, with Carlos Villar-Palasi, we next went on to demonstrate the intracellular activation of glycogen synthase by insulin [2]. Of the 4 enzymes of the glycogen cycle, glycogen synthase proved to be rate-limiting and specifically activated following insulin pretreatment of diaphragms [2,3]. Insulin activation of glycogen synthase was observed only when the enzyme was measured without added allosteric activator glucose 6-P. When glucose 6-P was added in excess to express "total" fully activated enzyme, no difference was detected in extracts between control and insulin pretreated diaphragms. UDPG pyrophosphorylase, phosphorylase, and phosphoglucomutase three other enzymes of glycogen synthesis and degradation (glycogen cycle) were non-rate-limiting and again no differences in these three enzyme activities were found in extracts between control and insulin pretreated diaphragms [2].

Based on these results, and in analogy with phosphorylase, we postulated two forms of glycogen synthase, an active form (active without added glucose 6-P) which was increased with insulin pretreatment [2]. An inactive form, (active only in the presence of added glucose 6-P) was correspondingly decreased with insulin pretreatment. Thus, insulin acted intracellularly to interconvert the inactive to the active form with no change in "total" actively.

Biochemically, with Rosell-Perez and Villar-Palasi, we next provided evidence in support of the hypothesis by demonstrating the existence of two forms [4]. We proved that they differed in covalent phosphorylation state. In contrast, to phosphorylase, the active form, termed I for independent was shown to be the dephosphorylated species, while the inactive form, termed D for dependent (on the presence of glucose 6-P in the assay) was shown to be phosphorylated. Both a phosphatase (Mg$^{++}$ stimulated) which activated glycogen synthase by dephosphorylation [5], and a kinase which inactivated by covalent phosphorylation were next demonstrated by Hizukuri and myself [6]. The kinase was shown to be stimulated by cAMP by Huijing and I and was termed glycogen synthase I kinase [7]. It was purified and separated from the already known kinase, phosphorylase b kinase [8]. Using commercially available
substrates casein and histone, Krebs and coworkers subsequently further purified and renamed this new kinase cAMP-dependent protein kinase [9].

**ISOLATION OF A PUTATIVE INSULIN MEDIATOR**

Since we knew that insulin added directly to glycogen synthase had no effect, we treated diaphragms with insulin and searched for effects on the interconverting enzymes, the phosphatase and kinase. With Shen and Villar-Palasi we found no significant effects on the phosphatase, but did find an inhibitory effect on the cAMP-kinase [10].

Both an inhibition and a desensitization to the activating effect of cAMP were observed (right-shifted dose response curve) [11]. With Guinovart and Lawrence, we further demonstrated that insulin acted on the cAMP-kinase to maintain the enzyme in its holoenzyme-inactive form [12]. The exact mechanism still remains to be elucidated, but might involve an altered phosphorylation state.

Using enhanced cAMP inhibition in extracts following insulin pretreatment in vivo as an assay, we began to search for an intracellular second messenger. We prepared boiled extracts of muscle and/or liver from rats injected with saline or insulin and compared their ability to inhibit cAMP-kinase. We found an enhanced inhibition in the insulin compared to the control extracts [13].

**SEPARATION OF 2 PUTATIVE INSULIN MEDIATORS**

Our first purification step following deproteinization by boiling and charcoal adsorption of nucleotides was sizing on a Sephadex G-25 column. We found the active fraction in a ninhydrin staining peak which we termed peak II, separate from the major low UV absorption peak termed peak I. The active material had an apparent MW of about 1000 [13].

Dr. Jarett in St. Louis had developed a system in which he could activate mitochondrial PDH in a fat cell homogenate by adding insulin. This was exciting because his experiments suggested that a transferable factor was needed for the activation. He was unable to activate cell membranes or mitochondria directly with added insulin. When he added insulin to the cell...
membranes however, he could take the resultant supernatant and add it back to mitochondria and activate PDH.

To test whether our heat stable cAMP-kinase inhibitory factor was related to Jarett's PDH stimulatory factor, we sent blinded samples of our G25 sephadex column fractions to St. Louis where they were tested on PDH. The same fraction (peak II) which inhibited cAMP-kinase also activated PDH. Jarett and Seals further went on to demonstrate that the factor activated the PDH phosphatase rather than inhibiting the PDH kinase [14].

We next setup both assays, PDH phosphatase and cAMP-kinase in our lab to determine whether both bioactivities could be separated or were present in the same molecule. Data obtained on a further sizing column, Sephadex G-15, indicated that the two activities were partially separated [15]. We then successfully separated the two by ion exchange. We adsorbed the boiled, charcoal-treated extracts on an AG1x8 anion exchange resin column and eluted at pH 2.0 and 1.3 with HCl. The pH 2.0 eluted fraction activated PDH phosphatase and the pH 1.3 eluted fraction inhibited the cAMP-protein kinase. The separate fractions were further purified by several TLC steps to single spot purity and chemically analyzed. The pH 1.3 fraction contained myo-inositol, glucosamine and neutral sugars including mannose and galactose suggesting that it had arisen from a GPI anchored lipid or protein. The pH 2.0 fraction contained D-chiro-inositol and galactosamine suggesting that it had arisen from a separate and novel GPI anchored precursor [16].

STRUCTURE DETERMINATION OF pH 2.0 PUTATIVE INSULIN MEDIATOR.

To establish the structure of these putative mediators, we switched from rat liver to beef liver to obtain larger quantities necessary for chemical analysis. From several 1 kg aliquots of beef liver we were able to obtain ~200 nmols for complete structure analysis of the pH 2.0 species. We are still studying and have not yet completed the structure of the pH 1.3 species.

Chemical degradation and 1 and 2D NMR experiments established the structure as a pseudo-disaccharide of pinitol, the 3-O-methyl ether of D-chiro-inositol and galactosamine. This novel structure was even more unusual in that it appeared to be associated with or to contain chelated Mn^{++}. The structure was further proven by chemical synthesis.
BIOLOGICAL ACTIVITY

Both the chemically synthesized and beef liver putative pH 2.0 species are active in vivo to decrease hyperglycemia in STZ-diabetic rats and to promote glycogen synthesis in diaphragm in vivo [17]. The synthetic pseudo-disaccharide is active in micromolar amounts whereas the putative mediator from rat or beef liver is active in nanomolar amounts. We think that the difference in bioactivity derives from the need of the synthetic pseudo-disaccharide to become activated perhaps by chelating Mn$^{++}$ in the body, a kinetically time limited process.

In vitro the natural putative mediator activated PDH phosphatase by sensitizing the enzyme to its required metal Mg$^{++}$. The Mg$^{++}$ dose response is left shifted such that lower concentrations of Mg$^{++}$ are required to activate in the presence of the putative mediator [18]. In fact, with sufficient putative mediator, bioactivity is observed at extremely low or zero Mg$^{++}$ [18]. Of interest, Denton and coworkers had previously demonstrated that insulin itself acts in the same way on fat segments [19]. Insulin action also results in a shift of the Mg$^{++}$ dose response to the left, sensitizing PDH phosphatase to its required metal Mg$^{++}$. The activated synthetic pseudo-disaccharide activates analogously, while the non-activated pseudo-disaccharide is inactive. A similar action is observed on phosphatase 2C, another member of the Mg$^{++}$ requiring phosphatase family [18,20]. Our hypothesis is that the putative mediator is able to insert its activated structure in or near the metal binding site of the enzyme and thus allosterically sensitize it to or replace the Mg$^{++}$ requirement. Further studies will be required to establish its detailed mechanism of action and the physiological significance of its effect on phosphatase 2C. It is of interest in this connection that in a preliminary report Ortmeyer et al. [21] have observed an activation of phosphatase 2C in monkey liver following insulin administration in vivo.

CONCLUSION

In summary, a downstream acting putative mediator from beef liver has been isolated and structure determined. It is an inositol glycan pseudo-disaccharide composed of pinitol and galactosamine. In vitro it activates PDH phosphatase like insulin by shifting its Mg$^{2+}$ dose response to the left. It similarly activates phosphatase 2C originally isolated from rat liver as a phosphoprotein phosphatase with specificity for glycogen synthase [20]. In vivo, infused in STZ-diabetic rats, it reduces hyperglycemia to euglycemia at doses comparable to insulin.
without producing hypoglycemia [22]. We feel, it is an excellent candidate for a true insulin mediator. Of clinical interest, decreased chiro-inositol in urine and tissues of type II diabetic subjects and first degree relatives as well as in monkeys correlates with the degree of insulin resistance [23,24,25].
REFERENCES


STUDIES ON NATURALLY OCCURRING SUBSTANCES FOR INHIBITORS OF GLYCOSIDASES

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ABSTRACT --- Inhibition of glycosidases has been of a number of potential therapeutic uses, including the treatment of cancer, diabetes and AIDS. Hot water extracts of plants and crude drugs were assayed the inhibitory activities to α-glucosidase (α-glu), β-glucosidase (β-glu), β-galactosidases (β-gal), and α/β-mannosidases (α/β-man). Several examples of new active substances are as follows:

- **Broussonetin A** (IC₅₀, 16 nM to β-gal), 0.3 μM to α-man),
- **Broussonetine B** (11 nm to β-gal, 0.29 μM to α-man),
- **Broussonetine C** (36 nM to β-gal, 0.32 μM to β-man),
- **D** (29 nM to β-gal, 0.34 μM to β-man),
- **E** (3.3 μM to α-glu), 55 nM to β-glu), 2 nm to β-gal, 23 nm to β-man),
- **F** (1.5 μM to α-glu, 10 nm to β-glu, 4 nM to β-gal, 28 nM to β-man).

The structures were elucidated on the basis of the results of the spectroscopic analysis and chemical reactions.

Key words --- glycosidase inhibitor, crude drug, plant, polyhydroxypiperidine, polyhydroxypyrrolidine, structural elucidation.
Introduction

Glycosidases are concerned in several important biological events, such as intestinal digestion of carbohydrates, the biosynthesis of glycoproteins, and the lysosomal catabolism of glycoconjugates.  

Therefore, the inhibition of glycosidases has been of a number of potential therapeutic uses, including the treatment of cancer, diabetes and AIDS.

Many naturally occurring inhibitors of the glycosidases have been reported and used as lead compounds for useful agents.

Nojirimycin has been isolated from *Streptomyces* spp. as an antibacterial agent and its deoxy compound, deoxynojirimycin(1) has been prepared in the course of the structural elucidation.

Moranoline(1) has been isolated from *Morus* spp. and identified to deoxynojirimycin. This has been clarified as an inhibitor of α-glucosidase.

Fogamine(2) has been isolated from *Fagopyrum esculentum* as an inhibitor of α-glucosidase in mouse gut, and its isomers and glucosides(3) have been isolated from *Xanthocercis zambesiaca*. Deoxymannojirimycin(4) has been isolated from *Lonchocarpus* sp. as moderate inhibitors of several α-mannosidases and a good inhibitor of mammalian α-fucosidase.

Deoxyglucuronojirimycin(5) has been isolated from *Baphia racemosa* as an inhibitor of human liver β-D-glucuronidase.

α- and β-Homonojirimycins(6, 7)
have been isolated from *Aglaonema treubii* as inhibitors of several glycosidases along with their congenors. Calystegine B₂(8) has been reported as potent inhibitors of β-glucosidase and β-galactosidase from *Physalis alkekengi* var. *franchetii* along with other calystegines (9-14). Castamospermine (15) has been isolated from *Castanospermum australe* as inhibitors of some glycosidases (14).

A pyrrolidine, 2, 5-dihydroxymethyl-3, 4-dihydroxy derivative (16), has been isolated as inhibitors of α- and β-glucosidases from *Derris elliptica* (Leguminosae). While the above compounds (1-15) are piperidine derivatives, these lead compounds are going to produce fruitful results of many piperidine, pyrrolidine and glucosamine derivatives (e.g. 17-20) as inhibitors of glycosidases by synthetic methods (16, 17, 18). Acarbose (21) (19) and voglibose (22) (20) are excellent examples of antidiabetics, which are used for control of blood glucose concentration in diabetics due to postprandial elevation after carbohydrate digestion. Glycoprotein processing α-glucosidase I and II and α-mannosidase II play important roles in complex immunoglucoprotein processing. Therefore, the inhibitors are being used as tools for clarifying immunomodulatory agents. Acarbose (21) 2(R), 5(R)-Bis(hydroxymethyl)-3(R), 4(R)-dihydroxy pyrrolidine (23) and homonojirimycin (24) have been
shown to inhibit glycoprotein processing $\alpha$-glucosidase I and $\alpha$-mannosidase I, respectively, \cite{12} and are expected as important tools.

Our studies

I and the coworkers isolated $(2S,3R)$-(-)-3-hydroxybaikain(I) from a toxic mushroom, Russula subnigricans in 2% yield (for dry weight), \cite{22} and transferred to deoxyidonojirimycin (II) in spite of galactonojirimycin (III) expected as an inhibitor of fucosidase. \cite{23} (Fig. 1)

![Chemical Conversion to II (III) From I.](image)

Since this incomplete work, we began to study for naturally occurring substances of inhibitors of glycosidases. We have attributed primarily to screening of the inhibitory activities with hot water extracts of almost 100 kinds of plants and crude drugs to $\alpha$-glucosidase ($\alpha$-glu). (Table I) After the isolation and the structural elucidation of the principles, we have bioassayed secondarily the inhibitory activities of the to other glycosidases such as $\beta$-glucosidase ($\beta$-glu), $\beta$-galactosidases ($\beta$-gal), and $\alpha/\beta$-mannosidases ($\alpha/\beta$-man).
### Table 1. Positive Results due to the Primary Screening Test.

<table>
<thead>
<tr>
<th>Tested Materials</th>
<th>Inhibition Rates(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Japanese Knotweed</td>
<td>93.4</td>
</tr>
<tr>
<td>Ginkgo</td>
<td>70.4</td>
</tr>
<tr>
<td>Astragalus Root</td>
<td>51.3</td>
</tr>
<tr>
<td>(Dryopteris lancera)</td>
<td>97.3</td>
</tr>
<tr>
<td>Japanese Chestnut Oak</td>
<td>68.4</td>
</tr>
<tr>
<td>(Broussonetia kazinoki)</td>
<td>94.7</td>
</tr>
<tr>
<td>Comfrey</td>
<td>85.5</td>
</tr>
<tr>
<td>Pomegranate, Leaves</td>
<td>97.5</td>
</tr>
<tr>
<td>White Clover</td>
<td>91.5</td>
</tr>
<tr>
<td>Shasta Daisy</td>
<td>91.4</td>
</tr>
<tr>
<td>Mulberry Bark</td>
<td>97.9</td>
</tr>
<tr>
<td>Onion(Allium cepa)</td>
<td>69.3</td>
</tr>
<tr>
<td>Common Jujube, Leaves</td>
<td>92.9</td>
</tr>
<tr>
<td>Silk Tree(Albizia julibrissin)</td>
<td>95.8</td>
</tr>
<tr>
<td>Bush Clover(Lespedeza Bicolor)</td>
<td>70.0</td>
</tr>
<tr>
<td>Daisy(Stenactis annus)</td>
<td>82.2</td>
</tr>
<tr>
<td>Poria Sclerotium</td>
<td>52.1</td>
</tr>
<tr>
<td>(Eupatorium fortunei)</td>
<td>54.0</td>
</tr>
<tr>
<td>Imperata Rhimome</td>
<td>40.0</td>
</tr>
<tr>
<td>Hoop-Coop Plant</td>
<td>57.7</td>
</tr>
<tr>
<td>(Osmorhiza aristata)</td>
<td>60.9</td>
</tr>
<tr>
<td>Sumac(Rhus trichocarps)</td>
<td>99.0</td>
</tr>
<tr>
<td>Chinese Strawberry Tree</td>
<td>93.4</td>
</tr>
<tr>
<td>Coix Seed</td>
<td>47.5</td>
</tr>
<tr>
<td>Scopolia Rhizome</td>
<td>53.0</td>
</tr>
</tbody>
</table>
Assay of Inhibition of Glycosidases

The reaction mixture consisted of 475 µl 0.1M phosphate buffer (pH 7.0), 250 µl 250 mM p-nitrophenyl-β-D-glucopyranoside and 250 µl α-glucosidase (a stock solution of 1.0 mg/ml in 10 mM phosphate buffer (pH 7.0) was diluted 40 times with the same buffer, pH 7.0, just before the assay), for the substances (25 µl of solutions of concentration, 200-0.1 mg/ml). After incubation for 20 min at 37 °C and interrupting the reaction by addition of 1 ml 0.2 M sodium carbonate, the amount of p-nitrophenol liberated was measured colorimetrically at 400 nm (OD test). The inhibition rates (%) were calculated from the formula:

\[ \frac{100 \times (\text{OD test} - \text{OD blank})}{\text{control OD test} - \text{control OD blank}} \]

IC50 values were obtained from the inhibition curves. Assays for β-glucosidase, β-galactosidase, α-mannosidase and β-mannosidase were carried out as above using p-nitropheryl-β-D-glucopyranoside, -β-D-galactopyranoside, -α-D-mannosidase and -β-D-mannopyranoside as substrates.

The Results

So far we have isolated as the principles and clarified their structures on the basis of the results of spectoscopic analyses and chemical conversions as follows: mesotrihydroxypiperidine (IV), 3α,4β,5α-tri hydroxypiperidine (V), 3β,4β,5α-tri hydroxy-piperidine (VI) from Eupatorium...
fortunel (Compositae). We have also isolated and identified the compound V as a principle from Gymnema sylvestra (Asclepiadaceae). We have isolated broussonetinine A (VII), B (VIII) and broussonetine A-I (IX-XVIII) as inhibitors of glycosidases from Broussonetia kazinoki and B. papyfera (Moraceae) and elucidated their structures. We have also obtained imperatine A (XIX) as the active substance from Imperata cylindrica (Gramineae) and elucidated the structure. (Fig. 2) The IC50 values of tested compounds are shown in Table 2.

Further studies of these active substances for other kinds of bioactivities such as antivirus, antimetastatic, antidiabetic, acaricides, immunomodulatory agents and so on are going on now.

Table 2. IC50 (µM) of Glycosidase Inhibitors

<table>
<thead>
<tr>
<th></th>
<th>α-glucosidase</th>
<th>β-glucosidase</th>
<th>α-mannosidase</th>
<th>β-mannosidase</th>
<th>β-galactosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV</td>
<td>3.70</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>1.54</td>
<td>0.51</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>1.88</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VII</td>
<td></td>
<td>0.30</td>
<td></td>
<td>0.016</td>
<td></td>
</tr>
<tr>
<td>VIII</td>
<td></td>
<td>0.29</td>
<td></td>
<td>0.011</td>
<td></td>
</tr>
<tr>
<td>XI</td>
<td></td>
<td></td>
<td>0.36</td>
<td>0.036</td>
<td></td>
</tr>
<tr>
<td>XII</td>
<td></td>
<td></td>
<td>0.34</td>
<td>0.029</td>
<td></td>
</tr>
<tr>
<td>XIII</td>
<td>3.30</td>
<td>0.055</td>
<td></td>
<td>0.023</td>
<td>0.002</td>
</tr>
<tr>
<td>XIV</td>
<td>1.50</td>
<td>0.010</td>
<td></td>
<td>0.028</td>
<td>0.004</td>
</tr>
<tr>
<td>XV</td>
<td>10</td>
<td>0.012</td>
<td></td>
<td>0.038</td>
<td>0.002</td>
</tr>
<tr>
<td>XVI</td>
<td>10</td>
<td>0.018</td>
<td></td>
<td>0.016</td>
<td>0.001</td>
</tr>
<tr>
<td>XVII</td>
<td>0.130</td>
<td>0.240</td>
<td></td>
<td>0.029</td>
<td></td>
</tr>
<tr>
<td>XIX</td>
<td>11.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 2 Structures of New Compounds
References and Notes


[8] Evans, S.V., Hayman, A.R., et al, "Lack of glycosidase inhibition by, and isolation from Xanthocericis zambesiaca(leguminosae) of, 4-O-(β-D-glu-


[16] Fleet, G.W.J., Nicholas, S.J., et al, "Potent competitive inhibition of \( \alpha \)-galactosidase and \( \alpha \)-glucosidase activity by 1,4-dideoxy-1,4-iminopentitols: syntheses of 1,4-dideoxy-1,4-imino-D-lyxitol and of both enantiomers of 1,4-dideoxy-1,4-iminoarabinitol", Tetrahedron Lett., 26, 3127-3130 (1985).


[23] Not yet reported.


MEANINGS OF GENERALLY EXISTING NATURAL COMPOUNDS

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[INTRODUCTION] Kampo medicine, a boiling water extract of several kinds of herbal medicines, has been widely used for the clinical treatments in Japan. This medicine was created in China and brought to Japan via Korean peninsula at 5th century and has followed an independent course of development in Japan. At the end of 16th century, western medicine was introduced from Holland as Rampo. Since then, traditional orthodox medicine has been called Kampo. Unfortunately, in 1895 this precious traditional medicine was abandoned by Meiji government and only European's medical methods were adopted officially. Thus the termination of Kampo continued until recently. Lately, adverse effects of western drugs have developed serious problems and also limitations of their efficacy have been discussed, especially on physiological regulation. Under these circumstances, in 1977 the Ministry of Health and Welfare of Japan approved the application of Kampo medicine for the clinical treatment, then about 80% of clinical doctors trained in western medicine are experienced in Kampo medicine. But a scientific knowledge of Kampo medicine is still lacking, so this precious properties is slighted by modern doctors.

Last twenty years, we have investigated the Kampo medicine chemically and pharmacologically to change this situation. In this report, I will discuss on the generally existing compounds in the plant kingdom such as phenolic compounds and polysaccharides, which are the major components of Kampo medicine and play a very important role on their biological activities.

[DETAILS]
A. Gallic acid

Gallic acid is widely distributed in the plant kingdom as it is or the major component of hydrolyzable tannin. In screening anti-cancer agents in traditional Chinese medicines, gallic acid was found to exhibit cytotoxicity against all cancer cells examined and different phenomena against normal cells (Figure 1a and 1b). Gallic acid shows a typical selective toxicity and was found to induce apoptosis in HL-60RG cells.

Effects of gallic acid was examined for the specific metastasis of P815 cells to liver in vivo experiment (Table 1). Apparently, i.p.injection of gallic acid resulted in the prolongation of life span.
B. Polysaccharide

In general, polysaccharide possess some effects on immune systems.

Ethanol-precipitates (EP) fraction, which was obtained by the addition of ethanol to decoction of Kampo medicine and mainly consisted of polysaccharide, showed the enhancement of NO production (Figure-2) and phagocytosis.
C. Characteristic Ingredient

Every medicinal plant contains characteristic ingredients, which exhibit physiological activities respectively and some of them have been used as medicine, such as ephedrine, atropine, glycyrrhizin etc. Table 2 shows the characteristic ingredients and their pharmacological activities of seven crude drugs which compose the famous Kampo medicine, Shosaiko-to.

Table 2 Pharmacological Effect of Identified Ingredients in SST

<table>
<thead>
<tr>
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<td>Intermediary</td>
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<td>Parasite</td>
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<td>Metabolism</td>
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<td>Glycometabolic</td>
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<td>Sedative</td>
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<td>Laxative</td>
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</table>

Table 3

<table>
<thead>
<tr>
<th>Primary Physiological Modulator</th>
<th>Food</th>
<th>Medicine Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macromolecule</td>
<td>Small molecule</td>
<td>Saponin, Phenolic, Alkaloid, Steroid, Phenyl propanoid, Terpenoid etc.</td>
</tr>
<tr>
<td>Polysaccharide, Lignin, Protein, Tannin (tanin), Fiber etc.</td>
<td>Carbohydrate, Lipid, Amino acid, Vitamin, Peptide, Metal, GA etc.</td>
<td></td>
</tr>
<tr>
<td>Reguration</td>
<td>Effects on system</td>
<td>Symptomatic action Analgesic, Cholangic, Antipyretic etc.</td>
</tr>
<tr>
<td>Immunity, Metabolism, Intestinal flora etc.</td>
<td>Endocrine, Autacoid, Immune, Enzyme etc.</td>
<td></td>
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<tr>
<td>Normalization effects</td>
<td>Medicinal effects</td>
<td></td>
</tr>
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[CLOSING] In this report, I have tried to explain the features of Kampo medicine, whose general concept is shown in table 3.

Generally existing natural compounds in medicinal plants are common with those of vegetables and tea. These compounds will assist the individual homeostasis working normal.

References

Quality Control and Standardization of Phytopharmaca

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The interest in complementary therapies has considerably increased in the last decade. Among them, phytotherapy plays an important role and phytomedicines make a remarkable contribution to the total pharmaceutical market. Preparations based on garlic, ginkgo, ginseng, and hawthorn are leading phytomedicines in various countries of central Europe. In Germany, the market share of phytomedicines is about 6%. Phytomedicines are well-established in the OTC market and they are often prescribed by general practitioners. As a consequence, they must comply with the same requirements as all other drugs; that is to say their effectiveness, safety and pharmaceutical quality have to be demonstrated and documented. Unlike synthetic drugs, plant-derived preparations represent multicomponent drugs which make great demands on the analyst. In the case of garlic, ginkgo, and hawthorn, quality control is especially demanding because the constituents are complex in chemical structure, number, and in the special case of garlic, also concerning stability. This presentation deals with some general aspects of quality requirements as well as with analytical methods related to research topics covered by our work.

The quality of phytomedicines as well as that of the crude plant material used for the production of extracts has to be guaranteed, because lack of quality can lead to under- and overdosage or to side effects due to contaminations, impurities, or falsifications. Quality control of phytomedicines comprises the following three subjects:

- clear botanical identification of the plant material used
- proof of absence or of contents below official limiting values of undesired toxic components and toxicologically significant impurities such as heavy metals, pesticides, radioactivity, bacteria, or fungi
- quantitative determination of active components or lead compounds

In addition, a demand for validation and control of all steps of the production process are indispensable in order to guarantee a high quality of the resulting products. It has to be demonstrated that new analytical methods meet the requirements of internationally accepted quality control guidelines.

The great variability of composition and concentrations of compounds in plant material, caused by different plant species, different climate, light and soil conditions, harvesting of different parts of the plant or at different stages of development, or by different drying and storage methods, have to be taken into consideration. Different industrial processing leads to further alterations of the final products. As a consequence, standardization of the plant material, in-process controls and final product controls are essential, and a clear and detailed declaration of the product is necessary. Standardization makes possible the production of a homogenous preparation from...
heterogeneous starting material. Since it ensures a steady content within a given range, it plays an important role for reproducibility of therapeutic effectiveness from batch to batch.

The concentration of compounds in a standardized extract is fixed by official monographs or by the manufacturers themselves; therefore, standardization of plants with known active compounds is not problematic. For plants of which the active principles are not yet known or plants containing constituents with low stability, such as garlic, standardization is made on lead compounds, but this is only a temporary solution.

Sulfur containing L-cysteine derivatives have been reported to be characteristic, genuine constituents of various Allium species. The S-alk(ene)l-L-cysteine sulfoxides, especially $\left(+\right)\text{-S-}$ allyl-L-cysteine sulfoxide (alliin), are precursors of a variety of more lipophilic products derived from enzymatic conversion, e.g. alliin to allicin by the alliinase after cell rupture and further transformation to ajoenes, vinylthiins or sulfides. Most of the previous chromatographic analyses tended to concentrate on the latter compounds because they are considered to be associated with the biological activity of garlic. In this case, the genuine cysteine sulfoxides and probably also the $\gamma$-glutamyl peptides act as prodrugs. The quantitation of these genuine compounds for the quality control is reasonable. Depending on the processing technique, various pharmaceutical products such as garlic powders, dry extracts, oil-macerates, or steam distillates arise. Their constituents represent the lipophilic conversion products mentioned above which complicate or even disable a rational quality control.

Other plants, such as ginkgo or hawthorn can raise problems since in both plants, two groups of constituents, namely flavonoids and terpene lactones or procyanidins, respectively, are considered to be the active principles. Therefore, it has to be decided on which group of components the extract shall be standardized. Standardization on different compounds can make a comparison of different products difficult or even impossible. Nevertheless, standardization is nowadays done on flavonoids and terpene lactones, such as in the case of ginkgo. With this approach the number and the variety of chemical structures are the limiting factor. HPLC methods requiring hydrolysis of the flavonoid glycosides have been, therefore, elaborated. This hydrolysis step is necessary due to the great number of flavonoid glycosides found in these plants. As a result we can propose simple and reproducible methods which allow us to quantify the corresponding aglycones. In the case of ginkgo extracts the main aglycones kaempferol, quercetin and isorhamnetin are determined; in the case of hawthorn extracts, quercetin and vitexin are determined. For the quantification of the terpene lactones in ginkgo, HPLC and GC methods are available while the assay of procyanidins in hawthorn remains still an unresolved problem.
COMPLEX TETRA AND PENTACYCLIC COUMARIN DERIVATIVES FROM THE GENUS ERIOSTEMON (RUTACEAE)

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ABSTRACT

A phytochemical investigation of the west Australian species *Eriostemon brucei* (Rutaceae) yielded 15 complex coumarins based on a 6-(or 8)-C-geranyl-5,7-dihydroxycoumarin precursor. Four of these compounds were confirmed as the previously reported bruceol, deoxybruceol, eriobrucinol and hydroxyeriobrucinol. Among the 11 novel compounds were 6, named the protobruceols, which probably represent an intermediate (tricyclic) state in the formation of the more complex coumarins. Of the remaining 5, two were regioisomers of eriobrucinol and another an isomer of hydroxyeriobrucinol. The final two compounds, named the pseudobruceols, represent new cyclisation patterns for the geranyl side-chain. Critical factors in the identification of these compounds are discussed. Subsequent studies on an east Australian species, *Eriostemon myoporoides* revealed a series of 7 further coumarins of this class based on a 2'-deoxybruceol skeleton and formed from a 6-C-farnesyl-5,7-dihydroxycoumarin precursor. At this point these compounds and unique to these two species.

Keywords: *Eriostemon brucei; Eriostemon myoporoides*, Rutaceae; 6-(or 8)-C-geranyl-(or farnesyl)-5,7-dihydroxycoumarin derivatives; structure elucidation; chemical taxonomy.
INTRODUCTION

The genus *Eriostemon* Sm. (Rutaceae) comprises some 30 species of perennial shrubs and subshrubs found only in semi-arid areas of Australia [1]. The first species to attract serious attention from natural products chemists was *Eriostemon brucei* F. Muell., which was studied by two groups during the 1960's and 1970's with the isolation of four very unusual 6-C-monoterpenyl-5,7-dioxycoumarins; namely, bruceol (1) [2], deoxybruceol (2) [3], eriobrucinol (3) [4] and hydroxyeriobrucinol (4) [4].

\[ \text{Figure 1} \]

![Structural formulas of bruceol (1), deoxybruceol (2), eriobrucinol (3), and hydroxyeriobrucinol (4).]

The biosynthesis of these unusual coumarin derivatives has never been resolved by experiment but can be assumed to occur through different cyclisation pathways that are plausible from a 6-geranyl-5,7-dioxycoumarin intermediate (Figure 1). Thus, bruceol would appear to be derived through the initial formation of a 7-hydroxy-5,6-angular pyranocoumarin in which the 6',7'-double bond of the geranyl attacks the C-7-oxygen of the coumarin through C-7', and the consequent positive charge at C-6' is neutralised through attack from C-1' of the 1',2'-double bond of the pyran. The new ion generated at C-2' is neutralised by substitution with OH.
By contrast in deoxybruceol the likely precursor is a 5-hydroxy-6,7-linear pyranocoumarin in which the 6',7'-double bond of the geranyl attacks the C-5 oxygen through C-7', and the resulting C-6, charge is neutralised through attack from C-1'. However, unlike bruceol, the final charge on C-2' is now neutralised through addition of H rather than OH. Eriobrucinol would appear to derive from the same linear pyranocoumarin intermediate as deoxybruceol, but in this case the additional cyclisation appears to be through a direct interaction between the 1',2' and 6',7' double bonds. The additional hydroxylation found in hydroxyeriobrucinol probably arises through a non-related subsequent modification.

Figure 1. Putative routes for the formation of bruceol (1), deoxybruceol (2) and eriobrucinol (3) from a 6-geranyl-5,7-dioxypyranocoumarin intermediate.

In 1990 we were fortunate to receive samples of both *E. brucei* and *E. brucei* subsp. *cinereascens* P. G. Wilson as part of our studies, in collaboration with Australian botanists, on the chemotaxonomy of Australian Rutaceae. Our investigation of these established that eriobrucinol and hydroxyeriobrucinol were the major coumarin metabolites together with smaller amounts of bruceol and deoxybruceol. As a result of this we were able to record, for
the first time, comprehensive NMR data for all four compounds [5, 6]. We were also able to isolate, as minor components, several further metabolites of this pathway [6, 7].

Six of these novel metabolites, to which we have assigned the trivial name protobruceols, are relatively simple pyranocoumarins. These are exemplified by protobruceol-II (5) and protobruceol-IV (6) [7]. It is noteworthy that the protobruceols represent the putative intermediate pyranocoumarin from which it is presumed that the more complex compounds such as bruceol and derived.

Five further tetra or pentacyclic coumarins were obtained as minor compounds in this study [6]. Two of these, isolated as very minor products, proved to have very similar $^1$H NMR spectral properties to eriobrucinol revealing the presence of a BCH-CH-CH$_2$-CH$_2$- spin system attributable to part of the geranyl side chain. A comprehensive series of NMR experiments including HC-COBI ($^1$J H-C) and HMBC ($^2$J and $^3$J H-C) allowed unambiguous assignment of all $^1$H and $^{13}$C chemical shift values. A striking feature of the $^1$H spectrum was the observation of long-range coupling over 5-bonds between H-4 and H-8 (Figure 2).

Figure 2. H-4 to H-8 long-range ($^3$J) coupling in coumarins.
This so-called Azig-zag\(^2\) coupling was observed in the spectrum of only one of the minor eriobrucinol-like compounds which implied that H-8 was unsubstituted in that compound. This led to the assignment of structure as \((7)\), which is the 5,6-angular homologue of eriobrucinol. This compound has been previously obtained as a by-product of the synthesis of eriobrucinol \([8]\) and is known as eriobrucinol regioisomer A. The proposed structure for eriobrucinol regioisomer A was also supported by a downfield shift of 0.13 ppm in H-8 compared to eriobrucinol, due to the presence of a free ortho hydroxyl.

The second eriobrucinol homologue lacked H-4/H-8 coupling and exhibited the resonance for the single aromatic proton at \(* 6.21\) as a sharp singlet shielded by 0.26 ppm compared to \(3\).

This compound must be the corresponding eriobrucinol regioisomer \(B\) \((8)\), which was also prepared synthetically by Crombie \textit{et al.} \([8]\). All of the eriobrucinols are characterised by widely non-equivalent resonances for the gem-dimethyl group in the \(^1\)H NMR spectrum \((0.66 \text{ ppm in } 3, \ 0.50 \text{ ppm in } 7, \ 0.77 \text{ ppm in } 8)\). This is presumed to be due to the proximity of one of the methyls to an oxygen \((C-5 \text{ in } 3, \ C-7 \text{ in } 7, \ C-9 \text{ in } 8)\). Eriobrucinol regioisomer B is distinct from all other complex coumarins obtained by requiring the formation of an 8-geranyl-5,7-dioxycoumarin precursor.

![Chemical structure of compounds 7 and 8](image)

The previously reported hydroxyeriobrucinol \((4)\) \([4]\) was identified in our material by a comprehensive NMR investigation \([6]\). The oxymethine proton (H-5') revealed only one significant coupling, which was to one of the H-4' protons, which necessitated zero coupling between H-5' and both the second H-4' proton and H-6' (Figure 3a). The relative stereochemistry of \(4\) was examined by means of a NOESY experiment which proved most
useful in confirming the all cis relationship between H-1', H-2', H-6', and the C-3' methyl (Figure 3b). Technically, we believe that the original name of hydroxyeriobrucinol is unsatisfactory and this compound should, in future, be referred to as 5'S-hydroxyeriobrucinol.

Figure 3. (a) Coupling constants (Hz) and (b) nOe interactions observed for 5'S-hydroxyeriobrucinol [6].

Another of the compounds isolated in our study proved to be isomeric with 4 and was identified as 4'S-hydroxyeriobrucinol (9) [6]. The HREIMS proved identical to 4 while the $^{13}$C NMR spectrum indicated the same compliment of carbon signals with significant variation observed only between C-2' and C-6' (Table 1). Analysis of the coupling constants and NOESY gave important information. The former (Figure 4a) showed the oxymethine proton to have two couplings while H-6' now coupled to an H-5'. Critical factors in the NOESY were the appearance of an H-5'S-H-6' interaction and the loss of the interaction between the C-3'-methyl and H-4'L (Figure 4b).

Table 1. $^{13}$C NMR chemical shift values for the monoterpenyl part of 4 and 9

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<th></th>
<th>C-1'</th>
<th>C-2'</th>
<th>C-3'</th>
<th>3'-Me</th>
<th>C-4'</th>
<th>C-5'</th>
<th>C-6'</th>
<th>7'-Me</th>
<th>7'-Me</th>
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<tr>
<td>4</td>
<td>36.2</td>
<td>38.3</td>
<td>85.3</td>
<td>29.6</td>
<td>48.4</td>
<td>73.1</td>
<td>56.8</td>
<td>38.7</td>
<td>18.7</td>
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<tr>
<td>9</td>
<td>36.5</td>
<td>37.0</td>
<td>86.2</td>
<td>21.8</td>
<td>77.1</td>
<td>34.3</td>
<td>42.2</td>
<td>39.8</td>
<td>18.5</td>
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</table>
Two further monoterpenyl coumarins proved to be somewhat different from the pentacyclic bruceol type compounds [6]. The NMR spectra for the first of these (HREIMS C_{10}H_{10}O_{6}) revealed the presence of an isopropenyl moiety, a -CH_{2}-CH_{2}-CH- spin system, two quaternary sp^{3} oxygenated carbons (96.3, 82.7 ppm), a methyl and a carbonyl (199.6 ppm). A sharp signal at δ 12.25 in the ^{1}H NMR spectrum required an H-bonded hydroxyl. The combined HC-COBI and HMBE experiments were able to establish the monoterpane fragment (9) and, given that there must be an aromatic hydroxyl on the coumarin moiety peri to the carbonyl for H-bonding to take place this compound must have structure 10, to which we assigned the trivial name pseudobruceol-I. A linear annulation for 10 was indicated from the resonance positions of H-4 and H-8.
The final complex coumarin again had all the normal characteristics of a monoterpenyl-5,7-dioxycoumarin but with the additional feature of a methoxyl substituent. This was readily placed at C-5 as it revealed an nOe interaction with H-4 of the coumarin and, additionally, with a methine proton which resonated at $^\ast$ 5.14. The NMR signals for the terpene component were markedly different from those of any of the other coumarins obtained; the $^1$H spectrum revealed a BCH$_2$-CH-O- spin system (with an equatorial oxymethine), a BCH$_2$-CH(O)-CH=C- spin system (axial oxymethine, and olefinic proton showing long range coupling to a methyl), and three deshielded methyls. The $^{13}$C spectrum revealed 3 $sp^3$ oxygen-bearing carbons (one quaternary) and a trisubstituted double bond. An HMBC experiment was very instructive and supported the assignment of structure (Figure 5a) while a NOESY experiments added information on relative stereochemistry (Figure 5b).

Figure 5. (a) HMBC correlations, and (b) nOe correlations for pseudobruceol-II (11)

On the basis of these experiments this compound was assigned structure 11, to which we have assigned the trivial name of pseudobruceol-II.
About two years after our studies on *E. brucei* we were able to carry out a similar investigation on an east Australian species, *E. myoporoides* DC. Previous to our work no coumarins had been reported from this species. From the aerial parts we isolated a total of 10 coumarins including the common scopoletin and scoparone. Seven of the other eight were pentacyclic coumarins analogous to bruceol but based on a 6-farnesyl-5,7-dioxycoumarin nucleus [9]. The 'H NMR spectra of each of the new coumarins showed 5,6,7-substitution of the coumarin nucleus while the $^{13}$C NMR chemical shift data confirmed 5,7-oxygenation and showed resonances for C-1', to C-10' of the terpene moiety (Table 2) that were similar to those for bruceol (1) [5] but without oxygenation at C-2'.

**Table 2.** Comparison of 13C NMR chemical shift data for bruceol (1) [5] and the most abundant of the *E. myoporoides* coumarins [9]

<table>
<thead>
<tr>
<th></th>
<th>C-1'</th>
<th>C-2'</th>
<th>C-3'</th>
<th>3'-Me</th>
<th>C-4'</th>
<th>C-5'</th>
<th>C-6'</th>
<th>C-7'</th>
<th>7'-Me</th>
<th>7'-Me</th>
</tr>
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<tbody>
<tr>
<td>bruceol</td>
<td>36.2</td>
<td>70.9</td>
<td>79.3</td>
<td>24.5</td>
<td>36.7</td>
<td>21.7</td>
<td>47.4</td>
<td>86.5</td>
<td>29.6</td>
<td>24.3</td>
</tr>
<tr>
<td><em>E. myop.</em></td>
<td>29.5</td>
<td>53.2</td>
<td>77.4</td>
<td>26.9</td>
<td>39.4</td>
<td>22.4</td>
<td>46.9</td>
<td>85.2</td>
<td>29.8</td>
<td>24.0</td>
</tr>
</tbody>
</table>

Each of the *E. myoporoides* coumarins gave HREIMS solving for C$_{26}$, which is five carbons more than bruceol. The additional 5 carbons were located at C-2' by means of HMBC experiments and the relative stereochemistry of the pentacyclic structure was shown to be
comparable to brucel by means of the NOESY technique. On this basis the common skeleton for these compounds was 12 with variation occurring only in the 2\(^\prime\) substituent (Figure 6).

\[ R = \begin{align*}
&\text{OH} \\
&\text{OOH}
\end{align*} \]

Figure 6. 6-C-sesquiterpenyl-5,7-dioxycoumarins from *Eriostemon myoporoides*.

One further coumarin of interest isolated from *E. myoporoides* was 8-geranyl-5,7-dihydroxycoumarin (13) [9]. This obviously has the potential to be the precursor of a series of 7,8-angular complex coumarins analogous to eriobrucinol stereoisomer B (6) but this does not happen; no cyclisation products could be found.
Wilson [1] divided the genus *Eriostemon* into seven sections and placed *E. myoporoides* and *E. brucei* in sections Erionema and Osmanthus respectively. He did this by raising Osmanthus as a new section whereas earlier taxonomic treatments had placed both taxa together in Erionema. We have now completed a study of some 15 of the 30-40 species of *Eriostemon* and while coumarins occur in most if not all species we have not encountered this type of compound other than in these two. The striking similarity of their coumarin metabolites certainly argues for a close relationship between them.
REFERENCES

ABSTRACT

The segmental method of quantifying steric effects has been previously applied to the determination of quantitative structure property relationships (QSPR) for chemical reactivities (rate and equilibrium constants), partition coefficients, cohesive energy densities, and chromatographic properties. It is here applied to bioactivities ranging from enzyme kinetic quantities to insect tissues and spinach chloroplasts. The method determines the locus of the steric effects in a substituent.

Keywords: Steric effects, segmental method, QSAR

THE NATURE OF STERIC EFFECTS

1.1 Primary steric effects - Primary steric effects result from repulsions between electrons in valence orbitals on atoms that are not bonded to each other. They are said to be due to the interpenetration of occupied orbitals on one atom by electrons on the other resulting in a violation of the Pauli exclusion principle. [1–4] All steric interactions raise the energy of the system in which they occur. They may decrease or increase a rate or equilibrium constant depending on whether steric interactions are greater in the reactant (increase) or the product (equilibria) or transition state (rate) (decrease).

1.2 Secondary steric effects - These effects are often due to the shielding of an active site from the attack of a reagent, from solvation, or both. They may also be due to a steric effect on the reacting conformation of a chemical species that determines its concentration.
1.3 Direct intramolecular steric effects - These effects result when the active site Y at which a measurable phenomenon occurs is in close proximity to the substituent X. Systems exhibiting direct intramolecular steric effects include ortho substituted benzenes, cis-substituted ethenes, ortho (1,2-, 2,1-; and 2,3-) and peri (1,8-) substituted naphthalenes, cis-1,2-disubstituted cyclopropanes, cis-2,3-disubstituted norbornanes, and cis-2,3-disubstituted [2.2.2]-bicyclooctanes. These effects may also occur in geminal systems, XCH₂Y; and in XY systems in which there is no skeletal group.

1.4 Indirect intramolecular steric effects - These effects occur when the steric effect of the variable substituent X is relayed by a constant substituent Z between it and the active site Y as in 1-Y-2-Z-3-XC₆H₆. This is a type of buttressing effect.

1.5 The directed nature of primary steric effects - Steric effects are vector quantities. Thus the pentyl and the 1,1-dimethylpropyl groups have the same volume but a different steric effect. That of the former is less than half that of the latter. In order to account for this consider the simple case of a spherical active site Y, in contact with a non-symmetric substituent, CZ²Z₄Z₅, where the superscripts, L, M, and S represent the largest, the medium sized and the smallest Z groups respectively. There are three possible conformations of this system; those in which Y is in contact with the Z²Z₅, Z²Z₄, and Z²Z₃ faces of the group respectively. As all steric interactions raise the energy of the system, the preferred conformation will be that which results in the lowest energy increase. This is the conformation which presents the smallest face to the active site, the Z²Z₃ face. That is the basis of the minimum steric interaction (MSI) principle: **A nonsymmetric substituent prefers the conformation that minimizes steric interactions.**

The MSI principal is essential to an understanding of steric effects. The vector property of steric effects means that group volume, a scalar quantity, cannot be a steric parameter. Group volumes are actually measures of group polarizability. **Steric effects are not directly related to bulk. Polarizability is.**
1.6 Intermolecular steric effects - Phenomena which depend on the difference in intermolecular forces between an initial and a final state frequently exhibit intermolecular steric effects. These phenomena often in the case of bioactivity often involve binding to a receptor site or to the active site of an enzyme. In the case of chromatographic properties binding to a surface may be involved. For such phenomena the greatest steric effect may occur at any position along the longest chain of the substituent (the group skeleton). It is important to note that while intramolecular steric effects result from proximity between substituent and active site on the same molecule intermolecular steric effects result from proximity between two different molecules. A substituent attached anywhere in the first molecule may interact with the second. The MSI principle continues to apply however.

THE MONOPARAMETRIC (MP) MODEL OF STERIC EFFECTS

2.1 Definition of the steric parameter - The hydrogen atom was chosen as the reference substituent and the steric parameter \( \psi \) defined as [1-4]:

\[
\psi = r_{vX} - r_{vH} = r_{vX} - 1.20 \quad (I)
\]

where \( r_{vX} \) and \( r_{vH} \) are the van der Waals radii (\( r \)) of the X and H groups in C units. All other measures of atomic size are a linear function of \( r \). There is no reason for preferring any measure of atomic size. As \( r \) are the best known and most frequently used measures of atomic size they are taken as the standard.

2.2 Steric classification of substituents - Substituents may be divided into three categories based on the degree of conformational dependence of their steric effects:

1. No conformational dependence (NCD). Groups of this type include monatomic substituents such as hydrogen and the halogens: cylindrical substituents such as the ethynyl and cyano groups, and tetracoordinate symmetric top substituents such as the methyl, trifluoromethyl and silyl groups.
2. Minimal conformational dependence (MCD). Among these groups are:

a. Nonsymmetric substituents with the structure \( MH_3(p) \) such as the hydroxyl and amino groups (\( p \) represents a lone pair).

b. Nonsymmetric substituents with the structure \( MZ^Z_L \), where \( S \) stands for small and \( L \) for large.

3. Strong conformational dependence (SCD). These groups have the structures:

a. \( MZ^Z_L \) and \( MZ^Z_LZ^Z \), where the superscript \( M \) indicates medium.

b. Planar \( \pi \)-bonded groups such as \( \text{Ph, carboxy} \).

c. Quasi planar \( \pi \)-bonded groups such as \( \text{NMe}_2 \) and cyclopropyl bonded to \( sp^2 \) hybridized carbon.

The steric parameter for NCD groups can be obtained directly from van der Waals radii or calculated from them. The values for SCD groups are often obtainable from van der Waals radii although in some cases they must be derived as secondary values from regression equations obtained by correlating rate constants with known values of the steric parameter. In the case of planar \( \pi \)-bonded groups the maximum and minimum values of the steric parameter are available from the van der Waals radii.

2.3 Planar \( \pi \)-bonded groups - These \( (X_{\mu}) \) groups represent an especially difficult problem because their delocalized electrical effect depends on the intramolecular steric effect when they are bonded to planar \( \pi \)-bonded skeletal groups, \( G_{\mu} \). The \( F_\alpha \) and \( F_\epsilon \), electrical effect parameters are a function of the dihedral angle formed by \( X_{\mu} \) and \( G_{\mu} \). The relationship used is:

\[
P = P_0 \cos^2 \theta
\]

where \( P \) is the property of interest, and \( P_0 \) is its value when the dihedral angle \( 2 \) is zero. Then:

\[
\sigma_{\alpha,\theta} = \sigma_{\alpha,\theta} \cos^2 \theta \quad (3) \quad \sigma_{\epsilon,\theta} = \sigma_{\epsilon,\theta} \cos^2 \theta \quad (4)
\]

where \( \sigma_{\alpha,\theta} \) and \( \sigma_{\epsilon,\theta} \) are the values of \( \sigma_\alpha \) and \( \sigma_\epsilon \) when the substituent and skeletal group are coplanar \( (2 = 0) \). The effective value of \( \psi \) is given by the expression.
\[ v = d \cos \theta + r_{ZS} - 1.20 \quad (5) \]

where \( Z \) is the smaller of the two \( Z \) groups attached to the central atom, \( M \) of the \( X^p \) group. There is no simple \textit{a priori} way to determine \( v \). It can be estimated by molecular mechanics calculations but there is some reason to believe that \( v \) is a function of the medium. Alternatively, the \( X^p \) group can be included in the data set by means of an iteration procedure. The method requires an initial correlation of the data set with all \( X^p \) and other SCD groups excluded. This constitutes the basis set. The correlation equation used for this purpose is the LDRS equation in the form:

\[ Q_x = L \sigma_{\alpha \varepsilon} + D \sigma_{\varepsilon \varepsilon} + R \sigma_{\alpha \alpha} + S \nu + h \quad (6) \]

The correlation is then repeated for each \( X^p \) group using \( L \) values increasing incrementally by some convenient amount from the minimum which represents the half-thickness of the group to the maximum which occurs when \( X^p \) is nearly perpendicular to \( G^p \). The proper value of \( v \) is that which:

1. Results in the best fit of the data to the correlation equation and
2. Has the \( L, D, R, S, \) and \( h \) values that are in best agreement with those of the basis set.

**MULTIPARAMETRIC MODELS OF STERIC EFFECTS**

When the active site is large and nonsymmetric or alternatively when the phenomenon studied is some form of bioactivity in which binding to a receptor is the key step, a monoparametric model of the steric effect is often insufficient. It is then necessary to make use of a multiparametric model of steric effects. Five multiparametric models are available; that of Verloop [5], the simple branching model, the expanded branching model, the segmental model, and the composite model [2,4]. The Verloop model suffers from the fact that its parameters measure maximum and minimum distances perpendicular to the group axis. These maxima and minima may occur at any point in the group skeleton (the longest chain in the group). The steric effect, however, may be very large at one segment of the chain and negligible at others. If a data set is large, the likelihood that the maximum and minimum distances of all groups are located at the same segment and that it is this segment at which the steric effect is important is
very small. The Verloop model will therefore not be considered.

3.1 The branching equations - The simple branching (SB) model for the steric effect is:

\[ S\psi = \sum_{i=1}^{n} a_i n_i + a_b n_b \]  

(7)

where: \( a_i \) and \( a_b \) are coefficients, \( n_i \) is the number of branches attached to the \( i \)-th atom, and \( n_b \) is the number of bonds between the first and last atoms of the group skeleton. \( n_b \) is a measure of group length. Unfortunately it is frequently highly collinear in group polarizability. For alicyclic substituents \( n_i \) is determined from an appropriate regression equation. For planar \( \pi \)-bonded groups \( n_i \) is taken to be 1 for each atom in the group skeleton. For other groups \( n_i \) is obtained simply by counting branches. The model makes the assumption that all of the branches attached to a skeleton atom are equivalent. **This is only a rough approximation.**

An improved model called the expanded branching (XB) equation:

\[ S\psi = \sum_{i=1}^{n} \sum_{j=1}^{n} a_{ij} n_{ij} + a_b n_b \]  

(8)

allows for the difference in steric effect that results from the order of branching. This difference is a natural result of the MSI principle. The first branch has the smallest steric effect because a conformation in which it is rotated out of the way of the active site is possible. This rotation becomes more difficult with the second branch and impossible with the third. The XB equation requires a large number of parameters. As few data sets are large enough to permit its use alternative models are necessary.

3.2 The composite model - This model, equation 9, is a combination of the MP model with the SB model. It has proven useful in modelling amino acid, peptide, and protein properties.

\[ S\psi = S\psi + \sum_{i=1}^{n} a_i n_i + a_b n_b \]  

\[ \psi_x = \sum_{i=1}^{n} \text{SIGMA from } j=1 \text{ to } m a_{ij} n_{ij} + a_b n_b \]  

(9)

The rationale behind this model depends on the relationship between the steric parameter \( \mu \) and the XB parameters given in equation 10 above. The \( \mu \) parameter is therefore a composite steric parameter of fixed composition. The SB term in the equation is a means of altering the
composition of the \( u \) steric parameter with a minimal number of parameters.

3.3 The segmental model - As both branching methods have problems associated with them, the segmental method is often the simplest and most effective. In this model each atom of the group skeleton together with the atoms attached to it constitutes a segment of the substituent. Applying the MSI principle, the segment is considered to have that conformation which presents its smallest face to the active site in the case of an intramolecular steric effect, or the receptor site, surface, or other molecule in the case of an intermolecular steric effect. The segment is assigned the \( u \) value of the group which it most resembles. Numbering starts from the first atom of the group skeleton; that is the atom which is attached to the rest of the species. In the absence of other effects the steric effect using the segmental model is

\[
S_{\varphi} = \sum_{i=1}^{n} S_i u_i \quad (11)
\]

In the case of chemical reactivity electrical effects occur and the LDRS equation is used. It is written as:

\[
Q_x = L \sigma_{\text{el}} + D \sigma_{\text{el}} + R \sigma_{\text{el}} + \sum_{i=1}^{n} S_i \nu_{\text{el}} + h \quad (12)
\]

where \( \sigma_{\text{el}} \), \( \sigma_{\text{el}} \), and \( \sigma_{\text{el}} \) are the localized (field), delocalized (resonance), and electronic demand electrical effect parameters [6,7]. In the case of the intermolecular force model the common form of the equation is:

\[
Q_x = L \sigma_{\text{el}} + D \sigma_{\text{el}} + R \sigma_{\text{el}} + M \mu_x + H_1 n_{\text{el}} + H_2 n_{\text{el}} + I I_x + A \alpha_x + \sum_{i=1}^{n} S_i \nu_{\text{el}} + B' \quad (13)
\]

Where the new parameters are the bond moment \( \mu \), polarizability \( \alpha \), hydrogen bond parameters \( n_{\text{el}} \) and \( n_{\text{el}} \), and ionic substituent parameter \( i \) [7].
APPLICATIONS OF THE SEGMENTAL MODEL

Correlations were carried out by multiple linear regression analysis. Electrical effect, dipole moment, hydrogen bonding and polarizability parameters were taken from or estimated as described in our earlier work [6-8]. Segmental steric parameters were obtained as described above.

4.1 Enzyme kinetics - Tsai [9] has reported kinetic parameters for ethanol oxidation and acetaldehyde reduction catalyzed by horse liver alcohol dehydrogenase (LADH) alkylated by the groups XCH₂ at Lyθ28 (sets 1 - 8). The equations used to describe the kinetics were for the oxidation and reduction:

\[ V_{\text{ox}} = V_l C_a C_b / (K_{i_a} K_b + K_a C_a + K_b C_b + C_a C_b) \]  (14)
\[ V_{\text{red}} = V_2 C_p C_q / (K_p K_q + K_q C_p + C_p C_q) \]  (15)

Where \( V_{\text{ox}} \) and \( V_{\text{red}} \) are the velocities, \( V_1 \) and \( V_2 \) are the respective maximum velocities, the C's are concentrations; A, B, P, and Q are NAD (nicotinamide adenosine dinucleotide); ethanol; acetaldehyde, and NADH (reduced NAD) respectively; \( K_{i_a} \), \( K_{i_b} \), \( K_p \), and \( K_q \) are Michaelis constants, and \( K_a \) and \( K_b \) are inhibition constants. Correlation with the equation:

\[ O_{\text{ox}} = L c_j + M X a - S, u x + S_2 v + S_3 a + B^o \]  (16)
gave as the best regression equations:

\[ \log V_{\text{ox}} = -2.32(±0.550) a_{\text{x}} - 0.495(±0.134) v, X + 2.27(±0.102) \]  (17)
\[ 100 R^2, 87.05; A100 R^2, 85.61; F, 26.89; S_{\text{est}}, 0.144; S^o, 0.422; n, 11. \]
\[ \log K_{i_a} = -2.78(±0.819) a_{\text{x}} - 0.444(±0.150) v, X + 0.616(±0.308) v, X + 2.55(±0.120) \]  (18)
\[ 100 R^2, 80.68; A100 R^2, 75.86; F, 9.747; S_{\text{est}}, 0.159; S^o, 0.551; n, 11. \]
\[ \log K_{i_b} = -1.78(±0.613) a_{\text{x}} - 0.314(±0.149) v, X + 0.670(±0.114) \]  (19)
\[ 100 R^2, 73.01; A100 R^2, 70.01; F, 10.82; S_{\text{est}}, 0.161; S^o, 0.607; n, 11. \]
\[ \log K_{p} = -2.66(±1.14) a_{\text{x}} + 0.639(±0.256) v, X - 1.57(±0.629) a_{\text{x}} - 0.521(±0.124) v, X + 0.417(±0.191) v, X + 2.70(±0.0747) \]  (20)
\[ 100 R^2, 92.88; A100 R^2, 88.13; F, 13.05; S_{\text{est}}, 0.0945; S^o, 0.396; n, 11. \]
\[
\log V_2 X = 5.68(\pm 2.10) \sigma_{00} - 1.39(\pm 0.430) \mu_X - 4.35(\pm 0.854) \nu_1 X + 2.45(\pm 0.131) \quad (21)
\]
\[
100 R^2, 84.55; A_100 R^2, 80.69; F, 12.77; S_{\text{av}}, 0.197; S^*, 0.493; n, 11.
\]
\[
\log K_{\text{av}} = 4.33(\pm 0.659) \sigma_{00} - 0.984(\pm 0.135) \mu_X - 2.79(\pm 0.268) \alpha_X + 0.284(\pm 0.0411) \quad (22)
\]
\[
100 R^2, 95.31; A_100 R^2, 94.13; F, 47.38; S_{\text{av}}, 0.0618; S^*, 0.272; n, 11.
\]
\[
\log K_{\text{av}} = 1.33(\pm 0.639) \sigma_{00} - 2.65(\pm 0.718) \alpha_X - 0.483(\pm 0.195) \nu_1 X + 1.74(\pm 0.139) \quad (23)
\]
\[
100 R^2, 78.97; A_100 R^2, 73.71; F, 8.760; S_{\text{av}}, 0.183; S^*, 0.575; n, 11.
\]
\[
\log K_{\text{av}} = -0.306(\pm 0.132) \nu_1 X - 0.481(\pm 0.176) \nu_1 X + 1.50(\pm 0.118) \quad (24)
\]
\[
100 R^2, 56.09; A_100 R^2, 51.21; F, 5.109; S_{\text{av}}, 0.151; S^*, 0.777; n, 11.
\]

The observed and calculated values of the bioactivity are given in Table 1 and zeroth order
partial correlation coefficients in Table 2 for all of the data sets studied in this work.

**Table 1.** Calculated and observed values for the data sets studied

<table>
<thead>
<tr>
<th>Set 1</th>
<th>X, log V_{l,0}, log V_{l,0}; H, 2.111, 2.250; Me, 2.029, 1.832; Et, 1.904, 1.874; Pr, 1.531, 1.689; tBu, 1.769, 1.650; Bu, 1.538, 1.570; tBu, 1.262, 1.221; MeCH=CH, 1.672, 1.685; PhCH=CH, 1.104, 1.185; Ph, 1.565, 1.407; CCl_3, 1.013, 1.136.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Set 2</td>
<td>X, log K_{l,0}, log K_{l,0}; H, 2.548, 2.554; Me, 2.248, 2.197; Et, 2.428, 2.386; Pr, 2.041, 2.258; tBu, 2.365, 2.287; Bu, 2.079, 2.127; tBu, 1.880, 1.809; MeCH=CH, 2.217, 2.278; PhCH=CH, 1.595, 1.731; Ph, 2.270, 1.977; CCl_3, 1.684, 1.752.</td>
</tr>
<tr>
<td>Set 3</td>
<td>X, log K_{l,0}, log K_{l,0}; H, 0.599, 0.670; Me, 0.320, 0.426; Et, 0.577, 0.342; Pr, 0.100, 0.260; tBu, 0.176, 0.177; Bu, 0.127, 0.177; tBu, 0.0101, 0.0488; MeCH=CH, 0.356, 0.252; PhCH=CH, -0.250, -0.0962; Ph, 0.312, 0.0601; CCl_3, -0.189, -0.102.</td>
</tr>
<tr>
<td>Set 4</td>
<td>X, log K_{l,0}, log K_{l,0}; H, 2.649, 2.700; Me, 2.467, 2.384; Et, 2.496, 2.527; Pr, 2.326, 2.454; tBu, 2.548, 2.489; Bu, 2.447, 2.381; tBu, 1.974, 2.006; MeCH=CH, 2.276, 2.243; PhCH=CH, 1.860, 1.919; Ph, 2.243, 2.178; CCl_3, 2.071, 2.078.</td>
</tr>
</tbody>
</table>
Set 5. $X$, log $V_{210}$, H, 2.483, 2.445; Me, 2.373, 2.188; Et, 1.914; Pr, 1.632, 1.784; iBu, 1.790, 1.579; Bu, 1.577, 1.579; tBu, 1.328, 1.579; MeCH=CH, 2.104, 2.255; PhCH=CH, 1.408, 1.434; Ph, 1.827, 1.555; CCl₃, 1.167, 1.221.

Set 6. $X$, log $K_{o2}$; H, 0.283, 0.284; Me, 0.107, 0.112; Et, 0.0212, -0.0187; Pr, -0.184, -0.147; iBu, -0.249, -0.278; Bu, -0.269, -0.278; tBu, -0.344, -0.278; MeCH=CH, 0.188, 0.211; PhCH=CH, -0.342, -0.294; Ph, -0.114, -0.237; CCl₃, -0.433, -0.413.

Set 1. $X$, log $K_{o2}$; H, 1.571, 1.743; Me, 1.604, 1.357; Et, 1.185, 1.232; Pr, 1.004, 1.110; iBu, 1.161, 0.986; Bu, 0.782, 0.986; tBu, 0.567, 0.638; MeCH=CH, 1.340, 1.203; PhCH=CH, 0.704, 0.764; Ph, 1.143, 0.984; CCl₃, 0.990, 1.049.

Set 8. $X$, log $K_{c2}$; H, 1.656, 1.499; Me, 1.281, 1.340; Et, 1.137, 1.340; Pr, 1.057, 1.090; iBu, 1.294, 1.090; Bu, 0.962, 1.090; tBu, 1.100, 1.120; MeCH=CH, 1.170, 1.075; PhCH=CH, 1.097, 1.051; Ph, 0.877, 1.051; CCl₃, 1.190, 1.077.

Set 9. $X$, log $K_{c2}$; H, -1.00, -0.782; Me, -0.638, -0.892; Et, -0.0915, 0.233; Pr, 0.719, 0.785; Bu, 0.740, 0.673; Pe, 0.679, 0.562; Hx, 0.748, 0.457; CH₂Bu, 2.301, 2.276; iBu, 1.322, 1.291; iPr, 0.320, 0.120; SEt, 1.523, 1.403; (CH₂)₂SEt, 0.813, 1.111; (CH₂)₃SEt, 0.930, 0.979.

Set 10. $X$, log $K_{c2}$; H, 0.301, 0.265; Et, 0.568, 0.699; Pr, 0.960, 0.914; Bu, 1.201, 0.914; Pe, 0.019, 0.914; CH₂Bu, 1.511, 1.547; iBu, 1.243, 1.131; iPr, 0.699, 0.699; SEt, 1.681, 1.799; C₂H₅, 1.690, 1.733; CH=CH₂, 1.000, 1.134; CH₂Cl, 1.550, 1.314; CH₂SEt, 1.550, 1.407; Ph, 1.523, 1.403; (CH₂)₂SEt, 0.813, 1.111; (CH₂)₃SEt, 0.930, 0.979.

Set 11. $X$, log $K_{c2}$; H, 0.301, 0.265; Et, 0.568, 0.699; Pr, 0.960, 0.914; Bu, 1.201, 0.914; Pe, 0.019, 0.914; CH₂Bu, 1.511, 1.547; iBu, 1.243, 1.131; iPr, 0.699, 0.699; SEt, 1.681, 1.799; C₂H₅, 1.690, 1.733; CH=CH₂, 1.000, 1.134; CH₂Cl, 1.550, 1.314; CH₂SEt, 1.550, 1.407; Ph, -0.194, 0.126; (CH₂)₂SEt, 0.178, 0.0750; (CH₂)₃SEt, 0.173, 0.120.

Set 12. $X$, p$K_{a2}$; H, 3.70, 3.96; F, 4.11, 4.01; Cl, 4.30, 4.47; Br, 4.89, 4.72; I, 5.16, 4.97; Me, 4.85, 5.19; Et, 5.32, 5.17; iPr, 6.47, 6.85; Bu, 6.40, 6.95; tBu, 6.80, 6.62; OMe, 4.66, 4.44; OEt, 5.22, 5.17; OPr, 4.80, 5.18; OiPr, 5.04, 5.18; OBu, 5.03, 5.18; OsBu, 5.15, 5.18; NMe₂, 5.10, 5.24; SMe, 5.15, 5.01; SPr, 5.96, 5.78; SiPr, 5.74, 5.78; SBU, 6.11, 5.78; SF₆, 4.85, 4.97.
Set 13. $X, pK_a, pK_w$.

- **H**: 3.70, 4.01
- **F**: 3.64, 3.53
- **Cl**: 3.62, 3.73
- **Br**: 3.85, 4.26
- **Me**: 4.00, 4.10
- **Et**: 4.42, 4.38
- **iPr**: 4.16, 4.48
- **tBu**: 5.74, 5.22
- **OMe**: 4.10, 3.42
- **OEt**: 4.16, 4.14
- **OPr**: 3.96, 4.34
- **OiPr**: 4.06, 4.34
- **OBu**: 4.70, 4.53
- **OsBu**: 4.49, 4.53
- **NMe$_2$**: 3.62, 3.91
- **SMe**: 4.47, 4.17
- **SPr**: 4.92, 5.10
- **SiPr**: 5.04, 5.10
- **SBu**: 5.52, 5.29
- **N0$_2$**: 2.52, 2.55

Set 14. $X, \log k$.

- **H**: 0.000, 0.000
- **Me**: -0.292, -0.346
- **Et**: -0.159, -0.0528
- **iPr**: 0.000, 0.000
- **Pr**: 0.301, 0.298
- **Pe**: 0.602, 0.585
- **CF$_3$**: 0.079, 0.088
- **MeOCH$_2$**: 0.079, 0.088
- **ClCH$_2$**: 0.301, 0.287
- **CH$_2$OH**: -0.046, -0.045
- **(CH$_2$)$_3$OH**: -0.079, -0.086
- **(CH$_3$)$_2$OH**: 0.519, 0.514

Set 15. $X, \log INH, \log I_{H}$. $H$: 1.23, 1.36

- **Me**: 1.43, 1.42
- **Et**: 1.53, 1.52
- **iPr**: 1.63, 1.53
- **tBu**: 1.60, 1.56
- **CF$_3$**: 1.85, 1.89
- **Cl**: 1.74, 1.66
- **OMe**: 1.38, 1.39
- **Ph**: 1.85, 1.86
- **3,5-Me$_2$**: 1.48, 1.49
- **3,5-Et$_2$**: 1.68, 1.69
- **3,5-iPr$_2$**: 1.73, 1.73
- **3,5-tBu$_2$**: 1.92, 1.93
- **3,5-(OMe)$_2$**: 1.43, 1.42

Set 16. $X, \log INH, \log I_{NH}$. $H$: 1.23, 1.23

- **Me**: 1.43, 1.43
- **Et**: 1.59, 1.58
- **iPr**: 1.66, 1.66
- **tBu**: 1.71, 1.74
- **Oc**: 1.97, 1.96
- **CF$_3$**: 1.82, 1.82
- **Cl**: 1.67, 1.66
- **Br**: 1.70, 1.71
- **OMe**: 1.23, 1.23
- **Ph**: 1.83, 1.83

Set 17. $X, pK_a, pK_w$.

- **H**: 5.53, 5.64
- **Me**: 5.91, 5.90
- **Et**: 6.10, 6.10
- **iPr**: 6.40, 6.10
- **tBu**: 6.04, 6.30
- **F**: 7.01, 7.13
- **Cl**: 7.27, 7.11
- **Br**: 7.18, 7.17
- **CF$_3$**: 7.31, 7.13
- **NO$_2$**: 6.45, 6.54
- **CN**: 6.68, 6.80
- **OMe**: 6.55, 5.61
- **OEt**: 6.19, 5.99
- **Ph**: 6.71, 6.72
- **N0$_2$**: 6.12, 6.32

Set 18. $X, pK_a, pK_w$.

- **H**: 6.55, 6.68
- **Me**: 7.07, 6.85
- **Et**: 6.74, 6.57
- **iPr**: 6.28, 6.59
- **tBu**: 6.28, 6.59
- **F**: 7.05, 7.02
- **Cl**: 7.23, 7.31
- **Br**: 7.34, 7.28
- **CF$_3$**: 7.25, 7.22
- **NO$_2$**: 6.57, 6.83
- **CN**: 6.70, 6.48
- **OMe**: 6.78, 6.84
- **OEt**: 6.37, 6.46
- **Ph**: 6.93, 6.91
- **N0$_2$**: 6.17, 6.15

Set 19. $X, pK_a, pK_w$.

- **H**: 5.7, 5.4
- **Me**: 6.3, 6.3
- **Et**: 6.5, 6.5
- **iPr**: 7.0, 7.4
- **tBu**: 7.0, 7.4
- **F**: 6.8, 6.9
- **Cl**: 6.7, 6.7
- **Br**, **Me**: 7.0, 7.1
- **Et**: 8.1, 8.0
- **NO$_2$**: 6.0, 6.1
- **OEt**: 7.1, 7.0
- **OMe**: 5.5, 5.6
- **OEt**: 6.7, 6.5
- **OBzl**: 7.4, 7.3
- **OBzl**: 8.1, 8.2
### Table 2. Zeroth order partial correlation coefficients.

<table>
<thead>
<tr>
<th>Sets</th>
<th>i, j, r, μ, σ, μ</th>
<th>ρ, ε, 0.950; ρ, α, 0.423; σ, v, 0.583; σ, u, 0.228; σ, v, 0.056; μ, α, 0.268; μ, v, 0.650; μ, v, 0.166; μ, v, 0.237; α, ρ, 0.418; α, ρ, 0.727; α, ρ, 0.605; ρ, u, 0.408; ρ, u, 0.258; ρ, v, 0.580.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Set 9</td>
<td>i, j, r, μ, σ, μ</td>
<td>ρ, α, 0.707; ρ, α, 0.203; σ, v, 0.180; σ, v, 0.005; σ, v, 0.134; μ, α, 0.095; μ, v, 0.095; μ, v, 0.069; μ, v, 0.112; α, ρ, 0.353; α, ρ, 0.536; α, ρ, 0.838; ρ, u, 0.434; ρ, u, 0.117; ρ, u, 0.429.</td>
</tr>
<tr>
<td>Sets 10, 11</td>
<td>i, j, r, μ, σ, μ</td>
<td>ρ, α, 0.210; σ, v, 0.140; σ, v, 0.005; σ, v, 0.185; μ, α, 0.285; μ, v, 0.123; μ, v, 0.066; μ, v, 0.139; α, ρ, 0.175; α, ρ, 0.404; α, ρ, 0.843; ρ, v, 0.060; ρ, v, 0.360; ρ, v, 0.355.</td>
</tr>
<tr>
<td>Set 12</td>
<td>i, j, r, μ, σ, μ</td>
<td>ρ, α, 0.274; σ, σ, 0.083; σ, μ, 0.895; σ, α, 0.133; σ, μ, 0.108; σ, ρ, 0.565; σ, v, 0.260; σ, v, 0.027; σ, σ, 0.494; σ, μ, 0.207; σ, α, 0.293; σ, u, 0.738; ρ, v, 0.491; σ, u, 0.470; σ, v, 0.476; σ, μ, 0.148; σ, α, 0.574; σ, u, 0.193; σ, v, 0.092; σ, v, 0.545; σ, v, 0.276; μ, α, 0.023; μ, v, 0.142; μ, v, 0.329; μ, v, 0.077; μ, v, 0.005; α, v, 0.165; α, v, 0.255; α, v, 0.688; α, v, 0.688; ρ, v, 0.481; ρ, v, 0.491; ρ, v, 0.488; v, v, 0.002; v, v, 0.275; v, v, 0.634.</td>
</tr>
<tr>
<td>Set 13</td>
<td>i, j, r, μ, σ, μ</td>
<td>ρ, α, 0.098; σ, σ, 0.058; σ, μ, 0.877; σ, α, 0.134; σ, μ, 0.276; σ, ρ, 0.137; σ, v, 0.224; σ, v, 0.102; σ, α, 0.394; σ, μ, 0.062; σ, α, 0.367; σ, v, 0.432; σ, u, 0.127; σ, v, 0.463; σ, v, 0.452; σ, μ, 0.196; σ, α, 0.576; σ, u, 0.172; σ, v, 0.313; σ, v, 0.553; σ, v, 0.237; μ, μ, 0.037; μ, μ, 0.422; μ, ρ, 0.076; μ, ρ, 0.040; μ, v, 0.081; μ, v, 0.101; α, v, 0.429; α, v, 0.678; α, v, 0.743; α, v, 0.530; μ, v, 0.460; μ, v, 0.368; v, v, 0.037; v, v, 0.086; v, v, 0.660.</td>
</tr>
<tr>
<td>Set 14</td>
<td>i, j, r, μ, σ, μ</td>
<td>ρ, α, 0.732; σ, α, 0.281; σ, v, 0.108; σ, v, 0.013; σ, v, 0.054; σ, v, 0.046; σ, v, 0.412; μ, α, 0.113; μ, v, 0.127; μ, v, 0.139; μ, v, 0.239; μ, v, 0.624; μ, v, 0.747; α, v, 0.486; α, v, 0.732; α, v, 0.729; α, v, 0.239; α, v, 0.182; v, v, 0.550; v, v, 0.130; v, v, 0.075; v, v, 0.075; v, v, 0.075; v, v, 0.075; v, v, 0.075; v, v, 0.075; v, v, 0.075; v, v, 0.075.</td>
</tr>
<tr>
<td>Set 15</td>
<td>i, j, r, μ, σ, σ</td>
<td>ρ, α, 0.554; σ, σ, 0.024; σ, μ, 0.902; σ, α, 0.284; σ, v, 0.408; σ, v, 0.132; σ, v, 0.218; σ, v, 0.061; σ, σ, 0.538; σ, μ, 0.505; σ, μ, 0.120; σ, v, 0.843; σ, v, 0.048; σ, v, 0.347; σ, v, 0.150; σ, μ, 0.010; σ, α, 0.601; σ, v, 0.551; σ, v, 0.231; σ, v, 0.720; σ, v, 0.720.</td>
</tr>
</tbody>
</table>
An important steric effect occurs at the first segment of the substituent in all cases except log \( K_p \). A dependence on \( \alpha \) occurs in all but log \( V_2 \) and log \( K_{a1} \), in the latter case collinearity with \( \mu \) and the poor results obtained for this set suggest that there may be a dependence on \( \alpha \) in this case as well. A dependence on \( \sigma_n \) and perhaps \( \mu \) as well seems to exist in the acetaldehyde reduction sets. There is a dependence on steric effect of the second substituent segment in log \( K_p \) and log \( K_{a1} \).
Jarv and coworkers [10] have reported second order rate constants $k_n$ and Michaelis constants $K_{cat}$ and $K_{app}$ for the hydrolysis of substituted alkyl acetates, XCH$_2$OAc, catalyzed by acetylcholinesterase (sets 9 - 11). The correlation equation is equation 17. The best regression equations are:

$$\log 10^{4} k_{n,X} = 0.298(\pm0.0592)\mu_x - 2.39(\pm1.29)\alpha_x + 2.38(\pm0.262)\nu_{2X}$$

$$+ 1.27(\pm0.394)\nu_{3X} - 0.782(\pm0.149)$$

(25)

$100 R^2, 92.18; A100 R^2, 90.62; F, 41.26; S, 0.257; S', 0.326; n, 19.$

$$\log 10^{2} K_{cat,X} = 3.28(\pm0.383)\sigma_X + 0.838(\pm0.173)\nu_{2X} + 0.414(\pm0.156)\nu_{3X} - 0.298(\pm0.104)$$

(26)

$100 R^2, 90.35; A100 R^2, 88.87; F, 37.46; S, 0.148; S', 0.359; n, 16.$

$$\log 10^{1} K_{app,X} = -1.00(\pm0.441)\sigma_X - 1.15(\pm0.199)\nu_{2X} - 0.938(\pm0.179)\nu_{3X} + 1.20(\pm0.120)$$

(27)

$100 R^2, 89.86; A100 R^2, 88.30; F, 35.46; S, 0.170; S', 0.368; n, 16.$

Significant steric effects are observed at the second and third segments of the substituent. There is a dependence on $\sigma_1$ or $\mu$ in all three data sets. There may be a dependence of $\log k_n$ on $\sigma, \sigma_1$, and $\mu$ are strongly collinear, $\alpha$ and $\nu_3$ less so.

Metcalfe and oukuto [11] reported $p_{50}$ values for fly head cholinesterase inhibition by 3- or 4-substituted phenyl N-methyl carbamates, XCH$_2$CONHMe (sets 12, 13). They were correlated with the equation:

$$Q_x = L\sigma_X + D\sigma_{ax} + R\sigma_{ax} + M\mu_X + A\alpha_X + H_1\pi_X + S_1\nu_{1X} + S_2\nu_{2X} + S_3\nu_{3X} + B'$$

(28)

The best regression equations for the 3- and 4- XCH$_2$CONHMe are:
$pI_{10} = -1.15(\pm0.441)\sigma_{\alpha} - 0.720(\pm0.106)\mu_{\alpha} - 2.58(\pm0.275)\nu_{\alpha}X$

$- 1.49(\pm0.281)\nu_{\alpha}X + 2.45(\pm0.131)$  \hspace{1cm} (29)

$100 R^2, 88.16; A_{100} R^2, 86.18; F, 31.63; S_{\alpha}, 0.294, S, 0.392; n, 22.$

$pI_{20} = -0.292(\pm0.441)\sigma_{\alpha} + 4.19(\pm1.46)\alpha - 0.241(\pm0.0956)n_{\alpha}$

$- 1.03(\pm0.467)\nu_{\alpha}X + 4.01(\pm0.181)$ \hspace{1cm} (30)

$100 R^2, 84.41; A_{100} R^2, 81.66; F, 21.69; S_{\alpha}, 0.312, S, 0.452; n, 21.$

Again, $\sigma_{\alpha}$ and $\mu_{\alpha}$ are strongly collinear, $\alpha$ and $\nu_{\alpha}$ less so. There is a dependence on steric effects at the third segment for both 3- and 4- $XCH_2CONHMe$. The 3-$XC,HCONHMe$ also show a steric effect at the first segment. Both sets also depend on $\mu$.

Inward and Jencks [12] have reported second order rate constants for the reaction of furoyl chymotrypsin with alcohols $XCH_2OH$ (set 14). Correlation with equation 16 gave as the best regression equation:

$log k_{2,3} = -3.05(\pm0.639)\sigma_{\alpha} - 0.104(\pm0.0234)\mu_{\alpha} - 6.24(\pm0.339)\mu_{\alpha} - 1.16(\pm0.0891)\nu_{\alpha}X$

$- 0.441(\pm0.0693)\nu_{\alpha}X - 0.000(\pm0.00256)$ \hspace{1cm} (31)

$100 R^2, 98.93; A_{100} R^2, 98.31; F, 111.6; S_{\alpha}, 0.0365, S, 0.147; n, 12.$

There is a dependence on steric effects at the first and third segments of the group and on $\alpha$, $\mu$, and $\sigma_{\alpha}$.

4.2 Other bioactivity data - Van den Berg et al. [13] have determined values for the inhibition of bovine serum albumin heat denaturation by 2-(3'- or 3',5'- and 4'-substituted phenyl)-1,3-indandiones (sets 15, 16). The data were correlated with equation 28. The best regression equations obtained for the 3'- and 4'- substituted compounds respectively were:
log $\text{INH}_x = 0.167(0.0216) \mu + 0.146(0.0213) \alpha + 0.185(0.0522) \nu_x$ 
+ 0.670(0.141) \nu_x + 1.36(0.0405) \quad (32)

$100 R^2, 89.62; A100 R^2, 86.78; F, 21.58; S_m, 0.0742; S', 0.395; n, 15.$

log $\text{INH}_x = -0.211(0.0521) \alpha + 1.24(0.225) \alpha + 0.169(0.00648) \mu + 1.40(0.0787) \alpha$
+ 0.302(0.0173) \nu_x - 0.159(0.0360) \nu_x + 1.36(0.0405) \quad (33)

$100 R^2, 99.81; A100 R^2, 99.62; F, 353.3; S_m, 0.0163; S', 0.0720; n, 11.$

Both sets show a steric effect at the second group segment, and a dependence on $\alpha$ and $\mu$. Set 15 shows a steric effect at the third segment while set 16 seems to be dependent on $\alpha$, $\beta$, and $\omega$ as well. The excellent fit of set 16 is almost certainly due to chance.

Fujita and coworkers [14] have measured pI$_{50}$ values for the inhibition of GluNAc incorporation into *Chilo suppressalis* Walker integument by 2-(4'-substituted phenyl)-5-(2',6'-dimethoxybenzoylamino)-thiadiazole in the absence (set 17) and presence (set 18) of piperonyl butoxide. The data sets were correlated with equation 28. The best regression equations for sets 17 and 18 respectively are:

\[
\begin{align*}
\text{pI}_{50} & = 2.48(0.244) \sigma_y - 0.476(0.0725) \alpha - 0.558(0.181) \nu_x \\
& + 0.824(0.250) \nu_x - 5.64(0.149) \quad (34)
\end{align*}
\]

$100 R^2, 92.77; A100 R^2, 90.80; F, 32.09; S_m, 0.187; S', 0.329; n, 15.$

\[
\begin{align*}
\text{pI}_{50} & = 3.39(0.749) \sigma_y - 0.706(0.286) \alpha - 9.05(3.69) \sigma_x - 0.674(0.163) \mu \\
& - 7.70(2.57) \alpha - 1.21(0.373) \nu_x - 6.68(0.175) \quad (35)
\end{align*}
\]

$100 R^2, 80.40; A100 R^2, 69.52; F, 5.17; S_m, 0.213; S', 0.606; n, 15.$

Both sets show a dependence on a steric effect at the first group segment and on $\sigma_y$.

Yoneyama and coworkers [15] have determined pI$_{50}$ values for Photosystem II inhibition of *Spinacia oleracea* L. chloroplasts by 5-RCO-3-(1'-(N-4'-substituted benzyl)-1'-R)-4-hydroxy-2-H-pyran-2,6-(3H)-diones where R is Me or Et (set 19). The data set was correlated with the equation:

\[
\begin{align*}
\text{Q}_x & = L \sigma_y - D \sigma_x + R \sigma_x + M \mu_x + H \eta_x + N \mu_x + S_1 \nu_x + S_2 \nu_x + R \eta_x + B'' \quad (36)
\end{align*}
\]

where $\text{Q}_x$ takes the value 1 when $R$ is Et and 0 when it is not. The best regression equation is:
Steric effects occur at the first and third group segments. There is a dependence on $c_1$ and $\alpha$.

CONCLUSION

We have shown that the segmental model of steric effects is applicable to both the simplest of bioactive substrates, enzymes, and also to more complex substrates such as protein (bovine serum albumin), spinach chloroplasts, and insects. The method is simple to use and the parameters are easily obtainable.
REFERENCES

ASYMMETRIC SYNTHESIS OF CARBOHYDRATES

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ABSTRACT

We have developed de novo asymmetric routes to 2-deoxyfuranosides (as precursors to nucleosides and modified nucleosides), using methodology which can allow for introduction of specific deuterium and $^{13}$C labels. We have also recently developed a de novo asymmetric method for the synthesis of six-carbon sugars. Specifically, this presentation will describe an enantioversatile route to D- and L-galactal, precursors to both D-galactose and L-fucose, by a de novo synthesis, using ethylbromoacetate (and its derived Wittig reagent), methylene Wittig, and DMF or paraformaldehyde, as sole carbon sources. A route to labelled propargyl alcohol has been developed in this work. The route is thus completely versatile with respect to introduction of any number and combination of $^{13}$C labels, since all these materials are commercially available labelled. Synthesis of an advanced intermediate di-$^{13}$C labelled is demonstrated. Additionally, the chemistry developed is designed to also allow for flexible introduction of regiospecific $^2$H labels. Our methodology should now allow for the targeted synthesis of specific $^{13}$C labelled monosaccharides for inclusion in target oligosaccharides, which will be useful for developing NMR techniques in the study of biologically important/therapeutically relevant carbohydrate-protein complexes.

Asymmetric synthesis of 2-deoxyfuranosides and nucleosides

2-Deoxyribofuranosides are precursors to nucleosides, and also to a variety of biologically active nucleoside analogues. The study of nucleic acid conformations and dynamics is of current importance to understanding issues with respect to DNA flexibility, DNA-protein binding, for example, as well as information regarding triple
helix formation by oligonucleotides with dsDNA targets. NMR is a tool to probe these phenomena. The use of stable labels greatly enhances the utility of nucleic acid NMR, with interests in $^{15}$N base labelling, but also additionally the inclusion of $^{13}$C labels in the sugar ring, or of specific deuterium labels. Both of these latter labelling options can provide valuable conformational parameters, $^{13}$C-labelling offers the possibility of editing techniques being employed, and both $^{13}$C and $^2$H offer opportunities to probe local dynamics.

We have been interested in de novo asymmetric synthesis as a versatile approach towards nucleoside analogues. We developed a short asymmetric synthesis of both D- and L-3'-azido, 3'-thio, 3'-seleno as well as d4 furanoside systems, and their derived nucleoside analogues. [1-3] This strategy, (Scheme 1), provides a more versatile route to intermediate allylic alcohol 4 than our initial route from crotonaldehyde. [11]

![Scheme 1](image)

Our synthesis is based on construction of the acetylene 3 which serves as precursor for reduction to the $\varepsilon$-alkene 4, and also to the $\zeta$ alkene isomer 7 using Lindlar’s catalyst.

Chirality for the carbohydrate is introduced then by Sharpless-Katsuki catalytic
asymmetric epoxidation, which proceeds in good to excellent yields, and with very high enantioselectivity in both enantiomeric series (traces of diastereomeric Moshers ester derivative observed just at detection limit on expanded 500Mz $^1$H NMR), providing 5 and 6.

The epoxy alcohols 5 and 6 undergo regioselective ring opening with a variety of nucleophiles, followed by selective acid catalysed ring closure to afford a range of furanosides. Critically, a wide range of Lewis acid catalysts proved unsuccessful in epoxide opening, and only the rarely used diethylaluminium fluoride proved versatile and selective enough to allow ring opening by a range of nucleophiles. Thus, azide, selenophenyl, hydride and alkoxide were all successfully employed. Except for opening with azide, each reaction also provided some of the alternate regioisomeric ring opening product. This was particularly pleasing since use of titanium tetraisopropoxide or titanium diazidodiisopropoxide, both previously reported, lead to substantial amounts of the regioisomer. Under milder conditions, titanium tetraisopropoxide led instead not to ring opening, but to the trimethylsilyl ether derivative of the epoxy alcohol in good yields.
The L-series sugars derived from 6 were also prepared analogously. These are of interest as some derived nucleosides show antiviral activity, and also the parent L-2-deoxynucleosides can be included in L-containing oligonucleotides (which show increased nuclease resistance), or homo-L-oligonucleotides which show strong binding to RNA.

Our methodology offers a variety of options for stable labelling. The acetylene functionality of 3 can be deuterated to provide the $^2\text{H}_2$-alkene ($\text{LiAl}^2\text{H}_4$ reaction, $^2\text{H}_2\text{O}$ quench) or the Z analogue (by using Lindlar's catalyst), or the regiospecifically mono-C3 deuterated $E$ isomer (by quenching $\text{LiAl}^2\text{H}_4$ reaction with $\text{H}_2\text{O}$) or mono-C4 deuterated compound ($\text{LiAlH}_4$ reaction, $^2\text{H}_2\text{O}$ quench). Additionally, use of deuteroparaformaldehyde allows for introduction of two $^2\text{H}$ labels at C5, or hydroxymethylation via formylation ($\text{Me}_2\text{N}^\text{13CHO}$) and reduction allows for $^{13}\text{C}$-$\text{C}_5$ labelling. $^{13}\text{C}$-labelling of the propargyl bromide can be envisaged using the chemistry we have recently developed for propargyl alcohol synthesis [described below]. Thus, the route developed is relatively short, enantiodivergent and allows for significant labelling versatility.

Alcohols 5 and 6 (protected) were also used as substrates for asymmetric dihydroxylations (AD), providing de novo synthesis of D- and L-xylofuranosides 17 and 18 (91% ee). Z-Alcohol 7 also provides a route using AD to ribofuranosides but in lower ee (30%).[4]

The above methodologies are the subject of a United States Patent issued in 1993. [5]
Asymmetric synthesis directed towards versatile stable labelling of D-galactose and L-fucose

A wide diversity of biological events involve carbohydrate-protein recognition events, and there is thus great interest in synthesis of oligosaccharides as tools for biochemical investigations, and as potential new therapeutic agents. [6,7] Oligosaccharide conformation is a critical determinant of these biological recognition phenomena which are involved in cell-cell signalling, adhesion, pathogen-cell recognition, chaperone-mediated protein folding, [8] antibody-oligosaccharide binding and tumour cell metastasis. Conformation and dynamics may be relevant to the influence of glycosylation on stability and function of enzymes for example. [9] Detailed structural analysis of carbohydrate-protein binding thus has extensive significance for understanding various biological events, and to potential new therapeutic opportunities including developing anti-metastatic drugs, carbohydrate vaccines, and anti-infectives or, alternatively, cell-targeting drug delivery vehicles. The 3D shape adopted by an oligosaccharide is determined largely by three torsion angles \( \psi, \phi, \psi \) (trans-glycosidic bonds) and \( \psi \) (involving C5-C6 bond). Determinations of free (unbound) oligosaccharide conformations is of importance (binding may select from free conformations or change them), and determination of protein-bound conformations with lectins and antibodies is becoming increasingly important. [10-12] The ultimate proof of bound sugar conformations can be provided by X-ray structures of the carbohydrate-bound complex, but this is not routine. Although there are a number of structures of protein-carbohydrate complexes there are only a handful of crystal structures of antibodies bound to carbohydrate epitopes. [13] Obtaining such structures is far from routine, and does not allow for dynamics studies.

There are a growing number of oligosaccharides whose recognition motif is known, and a number of others where structural, but not accurate functional, information is available. Examples of interest include disaccharide components of tumour antigens
including Galβ1→4Gal, Fucα1→2Gal and multigalactose components of GPI anchor structures.

![Glycosidic torsion angles](image)

**Figure 1:** Some potential $^{13}$C labelling sites $\text{JCC}$ and $\text{JCH}$ related.

NMR is the most powerful and versatile technique for study of free oligosaccharide conformations, through multidimensional homo- and heteronuclear methods. [14,15] Full $^1$H and $^{13}$C assignments are required, and several NMR techniques are routinely necessary to limit complications of extensive signal overlap in $^1$H NMR, and $^{13}$C spectra assignment difficulties. Conformational information is derived from inter-residue NOE constraints, and from coupling constants, particularly, $^3$J$_{\text{CH}}$ couplings involving the anomeric centre (trans-glycosidic link), which can provide information about $\jmath$ and $\varphi$, and $^1$J$_{\text{CH}}$ values, and $^3$H$_{\text{HH}}$ values involving C6/H6 which can provide information about $\omega$. However, NOE connectivities are more limited than for proteins, and the limited trans glycosidic coupling connectivity means that these data are rarely sufficient for direct conformational assignment. Additionally, in non-isotope enriched oligosaccharides, extensive signal overlap in $^1$H spectra complicates both assignments and coupling determinations, and $^{13}$C-$^1$H couplings can be difficult to observe in $^{13}$C spectra and unavailable in $^1$H spectra. Other types of experiments involving $^{13}$C nuclei are precluded by low natural abundance. Appropriate labelling strategies would greatly simplify assignments and analyses ($^{13}$C and $^2$H), and provide various options for obtaining further NMR parameters to assist direct conformational assignment as
well as facilitate methods not available at all in unenriched materials (including editing for bound carbohydrates).

$^{13}$C-labelling has already had some useful applications to determining carbohydrate torsional constraints from $J_{CH}$ couplings, [16-18] though is far from being fully exploited, and is some way from being routinely employed in oligosaccharide analysis. Development of new and modified NMR techniques through availability of new labelled materials offers improvements and new opportunities in oligosaccharide analysis, [19] (long-range heteronuclear techniques, $^{1}$H-$^{13}$C NOEs, new methods utilizing $^{13}$C-$^{13}$C couplings, $^{13}$C-$^{13}$C magnetization transfer etc., $^{13}$C-editing: 3D correlation spectra, impractical at natural abundance). NMR analysis of protein-bound conformations has seen some recent examples utilizing TRNOE experiments of weak-binding ligands (e.g. determining conformational changes of carbohydrate epitopes on antibody binding). In principle, use of $^{13}$C-labelled tight-binding ligands offers possibilities for experiments at concentrations typically appropriate to protein-ligand study.

Deuteration could serve purposes of simplifying 2D hetero and homonuclear spectra, and assist in assignments of both $^{1}$H and $^{13}$C spectra, and applications to dynamics studies are also possible. The potential utility of $^{2}$H-labelled monosaccharides for NMR investigations of oligosaccharides, has been limited by difficulty of synthesis of multideuterated monosaccharides from carbohydrates. Deuterated saccharides could also have applications to dynamic studies. Some perdeuterated sugars (various % incorporation) can be obtained biosynthetically, and some multideuterated systems are accessible by Raney nickel-catalysed exchange from D$_2$O, but specific designed patterns of regiospecific high percentage incorporation are not available. Introduction of specific $^{2}$H labels also allows use of $^{2}$H NMR (used in some glycolipid studies, potential for ligand-protein studies).
For extending the utility of carbohydrate NMR in these ways, availability of different multiply labelled monosaccharides is essential. However, relatively few $^{13}$C labelled hexopyranose sugars are available. Routes to several mono-$^{13}$C labelled monosaccharides have been described, \cite{18} employing $^{13}$C-labelled cyanide homologations of 5-carbon sugars. \cite{22} Synthesis of 1,3-di-labelled galactose and glucose have also been described in a similar way by iterative homologations of lower sugars. However, this method is lengthy, problematic for multiply labelled sugars involving several cycles of cyanide (labelled/unlabelled) additions, and isomeric separations at each iteration.

Current methods for introduction of $^2$H and $^{13}$C labels all have severe limitations for introducing specific $^{13}$C-labelling patterns, and for combined $^{13}$C/$^2$H-labelling. A versatile methodology for monosaccharides via a divergent de novo asymmetric synthesis could offer much more extensive labelling options and practicability. Few approaches to de novo asymmetric synthesis of hexopyranosides have been reported, \cite{23,24} and simple, general, strategies readily applicable to isotopically-labelled analogues are lacking.

D-Galactose and L-fucose are amongst the most common constituent monosaccharides of biologically important oligosaccharides. The strategy we have pursued is specifically designed to allow labelling versatility.

Retrosynthetically, a general strategy for asymmetric synthesis proceeds via immediate precursor polyols 20 and 21. Synthesis requires introduction of the four hydroxy centres with both absolute and relative stereocontrol, on a substrate with the two termini defined (to become the anomeric centre, and C6) $\text{[X=CH(OR)2 or CH}_2$OR].

The approach must be strategically economic (common versatile synthetic protocols), commence with readily available materials, and intermediates must be readily directed towards labelled analogues without the need for new steps. Most synthetic approaches
to monosaccharides involve diastereoselective homologation steps. The alternative is achiral construction of the carbon framework, with subsequent enantioselective then diastereoselective oxygenations. Constructing the 6-carbon chain through combination of one and two-carbon reagents is desirable as a range of simple $^{13}$C mono- and di-labelled compounds of this sort are available, providing maximum opportunity for isotopic diversity. LiAlD$_4$ and/or D$_2$O are relatively inexpensive deuterium sources, and thus routes using acetylene reductions, acidic exchange or protonation are most desirable for a versatile deuteration facility.

Osmium tetroxide dihydroxylation proceeds with exclusive syn diastereoselectivity. Substrate control of dihydroxylation of chiral allylic alcohols can provide a high degree of diastereoselectivity. Cis-allylic alcohols often show very high diastereoselectivity, and trans-disubstituted systems generally show good selectivity. [25] Trans-disubstituted alkenes are routinely excellent substrates for catalytic (21% of chiral ligand) asymmetric dihydroxylation (AD). [26] The two ligand series allow for both senses of enantiofacial additions with very similar enantioselectivity. (DHQD)$_2$PHAL and (DHQ)$_2$PHAL are commercially available at modest cost, and as mixtures with other reagent components as 'AD-mix a' and 'AD-mix b', and new variants (DHQD)$_2$PYR and (DHQ)$_2$PYR are now available (advantageous for certain alkene sub-classes). These ligands show essentially exclusive chemoselectivity in AD of electronically differentiated dienes (favouring more electron rich double bonds, as well as allowing mono-dihydroxylation of some dienes which are electronically similar), [27] and between double bonds and triple bonds. [28] The combination of the high enantio- and chemo/regio-selectivity of this methodology, the good, sometimes
essentially exclusive, substrate diastereocontrol expected for subsequent
dihydroxylation of appropriate AD-derived chiral allylic alcohol derivatives, or
matched double diastereoselection using a second AD, provides the technology
required for straightforward construction of enantiomeric monosaccharides from
achiral precursors.

L-Fucose and D-galactose share a common relative stereochemistry (differing only in
C6 OH), and thus should be accessible by parallel enantiomeric routes (either ligand
series are available), or alternatively by swapping the choice of terminus to become C1
within one enantiomeric synthesis. The C2 to C5 stereocentres consist of two pairs of
syn diols. The relative [anti] configuration between these pairs is suitable for substrate
control of introduction of the second diol function by dihydroxylation. Both L-fucose
and D-galactose systems are thus conceptually straightforward targets for the current
methodology (figure 2).

Our strategy is illustrated by the two complimentary retrosyntheses below, (for D-
galactose absolute stereochemistry if X=C1, and L-fucose if X=C6). The two routes
facilitate flexibility in construction of the carbon chain, and provide two options for
introducing the first diol (which may differ in e.e.s). Diols 22 or 25 could be obtained
from acetylenic diols 23 and 26, constructed via AD of the eneynes 24 and 27. Both
24 and 27 could be obtained from a common starting material 11. Ideally, this will
constitute a propargyl alcohol derivative (or an analogue at the aldehyde oxidation level). From the point of view of labelling, the use of propargyl alcohol (X=CH₂OH) derivatives is envisaged.

\[
\begin{align*}
&\text{22} & \text{23} & \text{24} \\
&\text{a} & \text{b} \\
&\text{25} & \text{26} & \text{27} & \text{28}
\end{align*}
\]

**Routes to enynes 1 - Wittig approaches:** We have prepared target enynes of type 24 and 27 in two ways, with both diethyl acetal and protecting alcohol termini. Thus, formylation of the acetylene 30 followed by Wittig reaction (with either Ph₃P=CHO or Ph₃P=CO₂Et), reduction and protection is successfully for synthesis of enynes 33. DMF (for formylation) and Ph₃P=CHCO₂Et are available with all possible ¹³C labelling options. Thus, labelling of propargyl alcohol or derivative would complete ¹³C-labelling versatility of this approach. The Wittig products were reduced in good yields, and the resulting allylic alcohols, after protection, underwent asymmetric dihydroxylation in 90-96% yields.
The diethylacetal bearing ynediols 35 proved essentially completely resistant to acetylene reduction with LAH, and thus we sought to evaluate analogous systems where both termini are at the alcohol oxidation level, but differentially protected.
Synthesis of Galactals: Propargyl alcohol derivatives were overall formylated in excellent yield, and pursuing the same Wittig homologation, reduction chemistry provided eneynes 38. These underwent asymmetric dihydroxylation and these diols 39 and 40 now underwent smooth reduction with LAH to enediol substrates. Diol protection and a second matching AD reaction in each case provided rapid access to the tetraol derivatives 43 and 44 establishes all carbohydrate functionality and stereochemistry. Choice of deprotection in each case ultimately decides whether the D-galactose or L-fucose (via L-galactose) route is pursued. We debenzylated both enantiomers. However, various direct oxidation methods (Swern, Dess-Martin, TEMPO) failed to cleanly effect aldehyde formation and the anticipated aldose cyclization to the sugars. Thus, the diol was acetylated, the terminal O debenzylated and then oxidized. Notably, using TBDMS and benzyl protected termini, LAH acetylene reduction was as problematic as in the case of the diethyl acetal terminus.

Routes to eneynes 2 - Coupling approaches: Eneyne 33 can also be obtained through Pd⁰ catalysed coupling. This approach employs all carbons derived from propargyl alcohol derivatives related to 28. While stannylation of propargyl alcohol itself or of simple ethers was poorly regioselective, and stannylation of propargylaldehyde diethyl acetal gave at best a 6:1 ratio of E/Z as main products 45 and 46, use of a higher order stannyl cuprate led to essentially quantitative stannylation with almost complete E selectivity in a remarkable reaction complete in less than 5 minutes. Coupling of the vinyl stannane 48 (from treating 45 with silica) with the iodoacetylene 49 proceeded in reasonable yields using bis(triphenylphoshine)palladium(II) chloride to afford eneynes 33.
Acetylene functionality allows for introduction of one (H2O work-up) or two (2H2O work-up) deuteriums via LiAlD4 reduction of the propargyl alcohols obtained by dihydroxylation. Higher degrees of deuteration are accessible via deuto-metallations of terminal (deutero)-acetylenes (deprotonation, 2H2O), or exchange reactions. A general route need only differentiate the terminal carbons with orthogonal protecting groups to allow labelled propargyl alcohol to serve as sole starting material, and thus 13C-labelling at any combination of sites is available by one synthesis of a 3-carbon synthet. A route to propargyl alcohol allowing 2H incorporation by exchange at the hydroxymethyl carbons allows C1 and/or C6 deuteration (C2 to C5 via acetylene reduction, above). No new chemistry is needed for any mixed labelled targets.
A route to labelled, protected propargyl alcohols 50 has been demonstrated starting from ethyl bromoacetate which is available either mono-$^{13}$C-labelled, or doubly-labelled. The methylene Wittig reagent required for homologation is also available $^{13}$C-labelled. Thus, this simple route allows preparation of any possible $^{13}$C-labelled analogue, including uniformly labelled! Deuterium could be introduced by exchange at ester or aldehyde stage. For synthesis of derived vinyl metals, the option of a terminal $^2$H would be available directly via work-up of the dibromide elimination reaction (via acetylide anion) with $^2$H$_2$O. Elimination of the dibromide derivative of 36 proved base and protecting group dependent, with competition from allene formation (exclusive product in some cases). The protecting group R=CHPh$_2$ proved most successful so far for acetylene 36 formation.

**Preliminary labelling:** One dilabelled intermediate diol, 53 has been prepared via the chemistry described above, which, by elaboration, is a precursor to either 4,5-$^{13}$C$_2$-D-galactose or 2,3-$^{13}$C$_2$-L-fucose.
REFERENCES

NEW PERSPECTIVES FOR THE TREATMENT OF HIV INFECTION (AIDS)
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B-3000 Leuven, Belgium

ABSTRACT

The recent launching of several new inhibitors of Human Immunodeficiency Virus (HIV) targeted at the HIV reverse transcriptase (RT) or HIV protease has led to the design of two-, three- or even four-drug combination trials. Initial evaluations have revealed marked reductions of viral load in patients receiving a triple combination of two nucleoside RT inhibitors with either a non-nucleoside RT inhibitor or protease inhibitor. However, whether the long-lasting suppression of viral replication will ultimately result in clinical improvement and prolonged survival, remains to be resolved. Namely, it is presently unclear whether multidrug-resistant HIV mutants will emerge after prolonged combination therapy. Cell culture studies should be useful to guide clinicians in choosing the optimal drug combinations. Also, inhibitors that are targeted at virus adsorption or fusion, or HIV integrase, have not yet been explored in clinical practice. Drug development is often complicated by a number of pharmaceutical drawbacks, namely: oral bioavailability, drug interactions, ease or cost of chemical synthesis, and convenience (and compliance) of the drug treatment regimens. It is concluded that all possible modalities to improve existing HIV therapies should be explored.

Key words: HIV, AIDS, reverse transcriptase inhibitor, protease inhibitor, triple-drug combination
INTRODUCTION

Since the discovery in 1983 of HIV as the etiologic agent of AIDS, the search for effective therapeutics has passed through several ups and downs. During recent years, the availability of a number of drugs with a favorable safety profile and with different modes of action, has allowed the design of two-, three- or four-drug combination schedules. Being initially introduced to increase therapeutic efficacy and safety, combination therapies may also markedly delay the emergence of drug-resistant viral mutants. However, the exceptional variability of HIV cautions against premature optimism, and underlines the need for new therapeutic agents which attack the virus at other sites in its replication cycle (Figure-1). Most agents that have been used in the clinic (whether under licensed form or in early trials) can be categorized as inhibitors of either virus adsorption, reverse transcription, or virus maturation. Insights in the processes of virus fusion or uncoating, integration, and translation are steadily growing and should ultimately lead to the development of new HIV inhibitors. In this respect, the recent findings that several chemokine receptors may act as coreceptors for the entry of HIV in the host cells, opens new possibilities for chemotherapeutic or immunotherapeutic intervention [1].

Here we will review the anti-HIV drugs that have already been or will soon be investigated in HIV-infected humans. A more detailed review of the individual compounds has been published earlier [2]. After a description of the individual therapeutic classes and their modes of action, we will highlight recent clinical data, with a special focus on drug combination studies and the development of drug-resistance. Finally, HIV therapy imposes a number of specific challenges to pharmaceutical research, and these will be commented on briefly.
INHIBITORS OF VIRUS ENTRY

Entry of HIV in the host cells (CD4-positive lymphocytes, or monocyte/macrophages) proceeds in three consecutive steps: adsorption, fusion, and uncoating. Adsorption of HIV to the cell membrane is directed by a specific interaction of the HIV gp120 envelope antigen with the CD4 molecule on the host cell. This interaction can be blocked by several polyanionic compounds, i.e., polysulfates [prototypes: dextran sulfate, pentosan polysulfate and poly(vinylalcohol sulfate)], polysulfonates [prototypes: suramin and poly(vinyl sulfonate)], polycarboxylates [prototype: poly(aurintricarboxylic acid)], polyoxometalates and polyphosphates [prototype: AR177 (Zintevir®)] [3]. These polyanionic compounds have been shown to interact with the positively charged amino acid side chains in the V3 loop of HIV gp120, thereby preventing the interaction of HIV gp120 with negatively charged surface molecules on the cell membrane. Thus, polyanions are not only able to prevent de novo infection of uninfected CD4-positive cells, but also cell-to-cell fusion between uninfected (CD4-positive) cells and infected cells, that express the HIV gp120 molecule on their membrane. The latter mechanism, called syncytium-formation, is considered to play a major
role in the gradual depletion of CD4-positive lymphocytes when HIV-infected individuals evolve to the symptomatic AIDS stage. Unfortunately, for a number of polyanionic compounds (such as dextran sulfate), systemic administration has been associated with severe thrombocytopenia due to the anticoagulant activity. On the other hand, the marked potential of dextran sulfate and related polyanions in preventing \textit{de novo} infection, makes these compounds ideally suited for topical use in vaginal microbicides, i.e., to prevent HIV infection during sexual intercourse [4].

\textbf{NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS (NRTIs)}

From the large number of 2',3'-dideoxynucleoside analogues that have been synthesized, five compounds have been formally licensed for use in HIV-infected patients, i.e., AZT, d4T, ddC, ddi and 3TC (Figure-2). The mode of action of these agents is based on their intracellular phosphorylation (by cellular nucleoside and nucleotide kinases) to the 5'-triphosphate form. The latter is structurally related to a natural nucleoside-5'-triphosphate, and therefore interacts with HIV reverse transcriptase (RT) by substrate inhibition and/or chain termination (because of the lack of a 3'-hydroxyl group in their sugar moiety). Consequently, the anti-HIV activity of these nucleoside analogues depends both on their affinity for the cellular kinases and their eventual interaction with the RT [5]. To partly overcome the inefficient phosphorylation of d4T and ddl, prodrugs have been designed in which the phosphorylated form (d4T-5'-monophosphate and ddA-5'-monophosphate, respectively) is attached to membrane-soluble lipophilic groups [6]. Another approach consists in the synthesis of chemically stable phosphonate derivatives of the nucleoside-5'-monophosphate, as demonstrated by the acyclic nucleoside phosphonate (ANP) analogues PMEA and PMPA (Figure-2) [7]. Compound 1592U89 is intracellularly converted to carbovir-5'-monophosphate, which is then further phosphorylated to carbovir-5'-triphosphate. This pathway prevents formation of free carbovir, which was previously shown to be associated with undesirable side effects.
The antiviral activity of the NRTIs is not restricted to HIV, but also extends to murine, feline or simian retroviruses, that infect mice, cats and monkeys, respectively. This allows the preclinical testing of NRTIs in appropriate animal models. Simian immunodeficiency virus (SIV)-infected monkeys are generally accepted as the most representative animal model for human AIDS. The SIV macaque model has been adapted to function as a model for specific clinical conditions, including parenteral virus inoculation (representing accidental needle stick contamination), vaginal virus inoculation (representing sexual transmission) and infection of newborns (representing perinatal transmission). Several NRTIs have been shown to be potent inhibitors of human hepatitis B virus (HBV). This is based on the reverse transcriptase function of the HBV DNA polymerase. Consequently, 3TC, PMEA and PMPA are also potential candidates for the treatment of HBV infections, for which no effective antiviral therapy (except for human interferon) is currently available [7,8].
Figure-3. Chemical structures and proprietary names of the non-nucleoside RT inhibitors (NNRTIs) that are commercially available or in advanced clinical trials.

NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS (NNRTIs)

Intensive screening programs has resulted in the identification of a large number of non-nucleoside RT inhibitors (NNRTIs), belonging to different chemical classes (Figure-3; Table-1). In contrast to the NRTIs described above, most NNRTIs are highly specific for HIV-1. The NNRTIs are non-substrate inhibitors and their inhibition of RT is non-competitive with regard to the natural substrate, and, as a result, the interaction is often highly reversible. The NNRTI-RT complex formation has been clearly demonstrated by crystallographic studies based on cocrystallization of HIV-1 RT with an NNRTI such as nevirapine (Figure-4) [9]. NNRTIs are generally assumed to bind to an hydrophobic pocket in which the YQYMDLY sequence (corresponding to amino acids 181–188) takes part. As a result, viral resistance to the NNRTIs
is most often associated with mutations in regions of the RT, that are surrounding the binding pocket such as amino acids 181–188 or 100–108 (Figure-4) [10]. HIV-1 mutants with decreased sensitivity to the NNRTIs emerge very rapidly, both in cell culture and in patients.

Table 1. Overview of NNRTI in various stages of development.

<table>
<thead>
<tr>
<th>Generic name (or abbreviation)</th>
<th>Chemical class</th>
<th>Company</th>
<th>Current status</th>
</tr>
</thead>
<tbody>
<tr>
<td>nevirapine (Viramune®)</td>
<td>dipyridiazepinone</td>
<td>Boehringer Ingelhein</td>
<td>licensed</td>
</tr>
<tr>
<td>delavirdine (Rescriptor®) (BHAP)</td>
<td>bis(heteroaryl)piperazine</td>
<td>Pharmacia &amp; Upjohn</td>
<td>licensed</td>
</tr>
<tr>
<td>tenofovir (TIBO)</td>
<td>tetrahydroimidazobenzodiazepinone</td>
<td>Janssen Pharmaceutica</td>
<td>Phase I</td>
</tr>
<tr>
<td>MKC-442 (HEPT)</td>
<td>hydroxyethoxymethylphenylthiodymine</td>
<td>Mitsubishi/Triangle Pharmaceuticals</td>
<td>Phase II</td>
</tr>
<tr>
<td>HBV 097</td>
<td>quinazoline</td>
<td>Hoechst Bayer</td>
<td>Phase II</td>
</tr>
<tr>
<td>pyridinone (L-697,661)</td>
<td>pyridinone</td>
<td>Merck &amp; Co</td>
<td>suspended</td>
</tr>
<tr>
<td>truvirdine (PETT)</td>
<td>phenylthiazolothiourea</td>
<td>Medivir/Abbott</td>
<td>preclinical</td>
</tr>
<tr>
<td>lestride (α-APA)</td>
<td>α-anilinophenylacetamide</td>
<td>Janssen Pharmaceutica</td>
<td>Phase II</td>
</tr>
<tr>
<td>DMP-266</td>
<td></td>
<td>DuPont Merck</td>
<td>Phase I</td>
</tr>
<tr>
<td>TSAO-T</td>
<td>tert-butyl-dimethyl-silylspirocine oxathiokisdione</td>
<td>–</td>
<td>preclinical</td>
</tr>
</tbody>
</table>

Due to its high degree of biological variation, HIV exists in the patient as a "quasispecies". From this mixture of variants, drug-resistant mutants can be easily selected following administration of an NNRTI. This is particularly prominent in cases where the mutation has no deleterious effect on the pathogenicity (i.e., survival capacities) of the viral mutant.

PROTEASE INHIBITORS

The HIV protease mediates the cleavage of the gag and gag-pol precursor proteins, and is therefore crucial in virus maturation when the new particles are budding from the cell (Figure-1). Inhibitors of this proteolysis are active in both acutely and chronically HIV-infected cells.
This feature gives the protease inhibitors an advantage over the compounds that inhibit early events such as virus entry or reverse transcription and that are only active in de novo infected cells. The first-generation protease inhibitors [i.e., saquinavir (Invirase®), indinavir (Crixivan®), ritonavir (Norvir®) and nelfinavir (Viracept®)], resulted from rational drug design based on the elucidation of the HIV protease structure, and the discovery of different cleavage sites that are unique to the HIV precursor proteins and are not present in normal mammalian proteins [11,12]. In contrast to these peptide-based protease inhibitors, most second-generation protease inhibitors are non-peptidic, resulting in a more favorable pharmacokinetic profile. Indeed, peptide-based protease inhibitors suffer from a number of handicaps, i.e., extensive first-pass metabolism, resulting in low oral bioavailability and short plasma half-life, considerable binding to plasma proteins, and poor penetration through the blood-brain barrier. The new HIV protease inhibitors include the peptidic compounds palinavir, KN1-272 and CGP 61755, and the non-peptidic compounds VX-478, U-103017, U-140690 and DMP-450 [13]. All these agents are very potent inhibitors of HIV protease in cell culture and in enzymatic studies. However, their clinical efficacy still needs to be proven.
MULTIPLE-DRUG COMBINATIONS: THE PANACEA AGAINST VIRUS RESISTANCE?

Until now, any HIV inhibitor used in monotherapy has been associated with the emergence of viral mutants that show reduced drug sensitivity. A list of more than 140 different mutations, that have been identified from cell culture or clinical isolation studies, is schematically given in reference [14]. The time to select for resistance and the underlying mutations differ markedly

**Figure-4.** Crystallographic structure of the HIV-1 RT p66/p51 heterodimer. The mutations at amino acid positions 41, 65, 67, 69, 70, 74, 75, 151, 184, 215 and 219 are characteristic of NRTIs. The mutations at amino acid positions 98, 100, 101, 103, 106, 108, 138, 179, 181, 188, 190 and 236 are characteristic of NNRTIs.
for (associated with 4 to 5 cumulative mutations) need several months to appear. For NNRTIs, resistance develops within only one or two weeks of cell culture. Note, however, that the levels of resistance and nature of the mutations observed in vitro, may not fully predict the clinical situation. It probably takes several weeks (or even months) for the drug-resistant mutant to overgrow the natural virus population, and to ultimately nullify the drug’s antiviral response. Nevertheless, there is general consensus that monotherapy should be avoided in the future, and that two- to three- (or even four-) drug combinations will be necessary to achieve full antiviral response and prevent rapid emergence of drug-resistant mutants [15]. It has been hypothesized that prolonged and total suppression of HIV replication, and hence, prevention of de novo infection of lymphocytes, could ultimately result in “eradication” of the virus, since the reservoir of HIV-infected lymphocytes may gradually shrink due to the limited life-time of mature lymphocytes. Whether this is a realistic objective depends on several factors. (1) The therapy should have a “knocking out” effect, i.e., virus replication should be annihilated to prevent resistance development. This “knocking out” effect could be achieved in cell culture if sufficiently high concentrations of NNRTI were used. (2) Drugs in the combination should act synergistically. Therefore, combinations of drugs with a different mode of action would be preferable. A typical example could be the combination of an NRTI + NNRTI + protease inhibitor. (3) These drugs have a different resistance profile. Multi-drug resistant HIV mutants have difficulties in arising when the combined mutations are either deleterious for viral pathogenicity, or when a mutation generating resistance against one drug results in hypersensitivity to another drug, or when a resistance mutation against one drug neutralizes resistance against another drug. (4) Alternating drug regimens, as initially proposed, should be completely avoided, since this results in the sequential accumulation of multiple mutations [16,17]. (5) Therapy should be started in the initial stage of disease, when the virus population has not yet become too heterogenous.
Table-2. Triple-drug combinations under clinical investigation.

<table>
<thead>
<tr>
<th>Drug combination</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>three NRTIs</strong></td>
<td></td>
</tr>
<tr>
<td>AZT + d4T + 3TC</td>
<td>18</td>
</tr>
<tr>
<td><strong>two NRTIs + one NNRTI</strong></td>
<td></td>
</tr>
<tr>
<td>AZT + d4T + nevirapine</td>
<td>19,20</td>
</tr>
<tr>
<td>AZT + d4T + delavirdine</td>
<td>21</td>
</tr>
<tr>
<td>AZT + ddC + delavirdine</td>
<td>21</td>
</tr>
<tr>
<td>AZT + 3TC + loviride</td>
<td>22</td>
</tr>
<tr>
<td><strong>two NRTIs + one PI</strong></td>
<td></td>
</tr>
<tr>
<td>AZT + ddC + saquinavir</td>
<td>11</td>
</tr>
<tr>
<td>AZT + ddC + ritonavir</td>
<td>23</td>
</tr>
<tr>
<td>AZT + 3TC + saquinavir</td>
<td>24</td>
</tr>
<tr>
<td>AZT + 3TC + ritonavir</td>
<td>25</td>
</tr>
<tr>
<td>AZT + 3TC + indinavir</td>
<td>25</td>
</tr>
</tbody>
</table>

NRTI: nucleoside RT inhibitor; NNRTI: non-nucleoside RT inhibitor; PI: protease inhibitor.

An overview of the triple-drug combinations that are currently under investigation is given in Table-2. For several of these drug combinations, interim analysis has shown marked and sustained suppression of plasma HIV RNA to levels below the detection limit. This was most prominent for the combinations of AZT plus 3TC plus a protease inhibitor such as saquinavir, ritonavir or indinavir.

PROBLEMS SPECIFIC TO PHARMACEUTICAL RESEARCH

The complexity of HIV infection enforces a multidisciplinary approach. A number of criteria specific to pharmaceutical development should be considered when designing new antiviral agents. (i) The two primary cell types infected by HIV are the T4-lymphocytes and monocyte/macrophages. Drugs should be active in both cell types, and should be able to reach infected cells in sanctuary sites such as the lymph nodes, in which distribution of the drug may be more difficult to achieve than in the general circulation. (ii) In addition, HIV has been found
to infect neuroglial cell lines, which could explain the neurological disorders that are frequently seen in AIDS patients. It is likely that HIV-infected macrophages transport the virus into the central nervous system (CNS) as they pass the blood-brain barrier. Treatment of neurological manifestations of HIV requires that the antiviral drugs are able to enter the brain. In the case of AZT, the lipophilic azido group facilitates its distribution to the CNS, and AZT therapy has indeed been associated with neurological improvement. (iii) Due to the chronic nature of HIV, long-term treatment appears to be unavoidable, and drugs should thus be preferentially administered by the oral route. For drugs that have low oral bioavailability, prodrugs can be designed that release the active drug after their first-pass. For instance, the low oral bioavailability of the ANP analogues PMEA and PMPA has been overcome by the use of their oral prodrugs bis(POM)-PMEA and bis(POC)-PMPA, respectively. (iv) Drug safety is a fundamental problem in a number of HIV-infected subgroups such as HIV-positive pregnant women and HIV-infected newborns. Assessment of embryotoxicity should therefore be an essential part of antiviral drug development. (v) The increasing use of triple-drug combinations raises questions about possible drug interactions. Special caution should be given to drugs that are extensively metabolized and may potentially induce liver enzymes, or compounds that are highly plasma protein-bound, and are thus subject to plasma protein displacement interactions. Other combinations may consist of drugs with a similar toxicity profile such as the NRTIs ddC and ddI, that can both be associated with peripheral neuropathy. A third site for drug interactions in AIDS patients relates to the frequent use of other drugs such as other antivirals, antibiotics, etc... (vi) The complex therapeutic schedules also impede patient compliance. Multi-drug preparations should therefore be considered, provided that the different drugs can be administered in a convenient drug treatment regimen (i.e., once or twice daily, with or without food, etc...). (vii) Finally, the costs of anti-HIV therapies are generally considered as prohibitive, making them mainly or exclusively accessible to wealthy populations. The implications of this evolution on our social security systems, and on the global health care situation, should receive more attention from both governmental and scientific authorities.
CONCLUDING REMARKS

During recent years, several new anti-HIV drugs, targeted at either the HIV reverse transcriptase or protease, have become commercially available. The different classes of drugs (NRTIs, NNRTIs, or protease inhibitors) differ markedly in their resistance profile (i.e., time to develop resistance and location of the responsible mutations). Thus, the remarkable therapeutic success seen with triple-drug combinations may be ascribed to a synergistic activity of drugs that act on different viral targets, resulting in a marked or even complete suppression of virus replication, and, consequently, prevention of resistance development. Whether the virus will be able to escape drug therapy (due to, for instance, virus reactivation from sanctuary sites such as the central nervous system or the lymph nodes) remains a critical question. Therefore, the search for agents that act on virus adsorption or fusion, or HIV integrase, or any other molecular target of the HIV replication cycle, should still be continued. It is obvious that basic research on the HIV replicative machinery and its intimate interaction with the host’s immune system may also lead to new therapeutic strategies.
REFERENCES


DESIGN AND SYNTHESIS OF TRICYCLIC NUCLEOSIDES (DIMENSIONAL PROBES) AS ANALOGS OF CERTAIN ANTIVIRAL POLYHALOGENATED BENZIMIDAZOLE NUCLEOSIDES.

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ABSTRACT: Structure activity relationship studies involving nucleosides that are structurally related to 2,5,6-trichloro-1-(β-D-ribofuranosyl)benzimidazole are presented. The relationship between these studies and the rationale for the design and synthesis of naptho[2,3-J]imidazole nucleosides are also presented. Several retro-synthesis of the requisite heterocyclic compounds as well as the corresponding nucleosides are described. Studies involved in the assignment of regiochemistry as well as stereochemistry is presented for all of the target (2-chloro, 2-benzylthio, 2-hydrogen) naptho[2,3-J]imidazole nucleosides. All of the target nucleosides have been evaluated for their anti-viral activity against human cytomegalovirus and toxicity against human foreskin fibroblasts cells.

Human cytomegalovirus (HCMV) is one of eight human herpes viruses. It is estimated that by adulthood, more than half of all Americans will have been infected with HCMV. HCMV infections in immunocompetent individuals are usually asymptomatic. However, in immunocompromised patients, HCMV infections are often life threatening. Transplant recipients and individuals with acquired immunodeficiency syndrome (AIDS) are particularly vulnerable to these infections. HCMV is also a leading cause of birth defects as a consequence of fetal infection in utero. Currently, there are three FDA-approved drugs available for the treatment of HCMV infections: ganciclovir (1), foscarnet (2) and cidofovir (3). Unfortunately, all three drugs can produce significant side effects and have limited oral bioavailability. Moreover virus strains
resistant to each of these drugs are emerging. Consequently, there is a need for a more potent and selective antiviral drug to treat HCMV infections.

As part of our search for new anti-cancer and antiviral drugs, we have prepared and evaluated a large number of compounds. We found that a number of benzimidazole nucleosides which had been previously synthesized possessed some antiviral activity. It was found that 2,5,6-trichloro-1-(b-D-ribofuranosyl)benzimidazole (TCRB, 4) (Figure 2), possessed very potent activity against HCMV with low cellular toxicity at concentrations which inhibit viral growth.
Biological evaluation of TCRB has established that its antiviral activity does not involve the inhibition of DNA, RNA or protein synthesis.\textsuperscript{15,18} It appears to act by a unique mechanism, which involves the inhibition of viral DNA processing and virus assembly,\textsuperscript{19-22} but the exact viral target is unknown. Structure activity relationship studies involving derivatives of 4, modified either in the heterocycle or carbohydrate moiety (Figure 2) have been undertaken in our laboratory,\textsuperscript{12-16,23-26} and evaluated for their biological activity. This structure activity relationship study involving changes on TCRB, while maintaining the basic integrity of the heterocyclic ring system, will be presented.

In an effort to expand on this basic structure activity relationship study, we have initiated an investigation into the spatial limitations of the targeted enzymes as it applies to the heterocyclic aglycone of TCRB. In this context, we have designed a series of tricyclic nucleosides as dimensional probes. These tricyclic nucleosides (I, II, III) were designed to effectively separate the benzene ring and the imidazole ring of TCRB in a linear fashion, in this instance a benzene ring was selected to function as the spacer. This selection was based on the fact that the benzene ring should function as a "pure" dimensional spacer since it would exert a minimal disturbance on the electron distribution of the parent ring system.\textsuperscript{27} In order to synthesize these tricyclic nucleosides, 2-substituted 6,7-dichloronaphtho[2,3-\text{-}]imidazoles were required as the heterocyclic intermediates. The most logical candidates were 6,7-dichloronaphtho[2,3-\text{-}]imidazo[2,3-\text{-}]imidazoles, 6,7-dichloro-2-methylthionaphtho[2,3-\text{-}]imidazoles or 2,6,7-trichloronaphtho[2,3-\text{-}]imidazoles (Figure 3). Therefore, a \textit{de novo} synthesis of these 6,7-dichloronaphtho[2,3-\text{-}]imidazoles was required.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Figure 3}
\end{figure}
In our first synthetic design, a sequential Friedel-Crafts reaction between imidazole derivatives and 4,5-dichlorophthalic anhydride was chosen to construct 6,7-dichloronaphtho-[2,3-j]imidazol-2-one (Scheme 1). However, as a general rule, imidazoles are resistant to a Friedel-Crafts reaction due to a marked deactivation in the presence of a Lewis acid. Imidazolin-2-one (13, 1,3-dihydro-2H-imidazole-2-one) appears to be an exception and apparently possesses sufficient activity towards an electrophile under Friedel-Craft conditions. 4,5-Dichlorophthalic anhydride (12) is commercially available and also easy to prepare from 4,5-dichlorophthalic acid.

A Friedel-Crafts reaction between 12 and 13 was conducted with anhydrous aluminum chloride in dry nitrobenzene. A number of unsuccessful attempts established that there are major difficulties associated with this reaction. Due to the inefficiency of the reaction and the difficulties we experienced in the subsequent work-up, this synthetic route was not pursued.

With the failure of the Friedel-Crafts reaction on 13, we initiated a route wherein the imidazole ring would be incorporated in the final stage of our synthesis. This approach was dependent on the availability of 2,3-diamino-6,7-dichloronaphthalene (14).
In our initial approach toward the synthesis of 14, a retro-synthetic route was selected based on a Diels-Alder reaction between 3,4-dichlorothiophene-1,1-dioxide (18) and benzoquinone (19) followed by a chlorination and amination to give the 1,4-naphthoquinone derivative (15) (Scheme 2). The key step in this route was the proposed reductive deoxygenation of 15 to afford 6,7-dichloro-2,3-diaminonaphthalene (14). Before initiating our research, we first studied some of the reductive deoxygenation reactions on 1,4-naphthoquinone. When 1,4-naphthoquinone was treated with hydroiodic acid in acetic acid at reflux temperature, we did not obtain any naphthalene. In an alternative approach, 1,4-naphthoquinone was first reduced to hydroquinone and then converted to the acetyl ester. Once again, naphthalene was not obtained when this ester was reduced under catalytic hydrogenation conditions. Considering that it would be even more difficult to effect a reductive deoxygenation on our highly functionalized 1,4-naphthoquinone 15, we decided to abandon this route.

We then investigated a Diels-Alder reaction between 4,5-dichloro-o-quinodimethane (23) and an appropriately substituted alkene, such as ethyl (E)-3-nitroacrylate (22) (Scheme 3).
The resulting 1,2,3,4-tetrahydro-tetra-b-substituted naphthalene 21 could then be aromatized to give the tetra-b-substituted naphthalene (20). Ethyl (E)-3-nitroacrylate (22) was chosen as the dienophile for the Diels-Alder reaction for the following reasons: 1) it can be easily obtained from ethyl acrylate in two steps and it is a good dienophile; 2) the ortho-located nitro group and ethoxycarbonyl group would be versatile functional groups for a construction of the imidazole ring.

A variety of methods for the generation of o-quinodimethanes have been reported. We elected to investigate the one pot method, first using different reaction conditions such as solvents, time of addition, time of heating and order of addition (Scheme 4, Method II). The best result was obtained when a suspension of the anthranilic acid (24) in vinyl acetate was added to solution of isoamyl nitrite in vinyl acetate at gentle reflux. However, this only gave a 6.7% yield of 1-acetoxy-4,5-dichlorobenzocyclobutene (26).
If the long time and high temperature required for the diazotization of 24 caused the low yield of 26 by Method II, then the first method\textsuperscript{20-44} should allow us to isolate the corresponding benzenediazonium-2-carbonate at a lower temperature (Scheme 4, Method I). However, it was found that this method was not suitable for the preparation of 25 because of the poor solubility of 25 in water.

The conversion of sulfones to \(\alpha\)-quinodimethanes requires a temperature above 200°C. On the other hand, sulfinates undergo decomposition of \(\alpha\)-quinodimethanes at a temperature around 80°C and can be easily prepared from the \(\alpha,\alpha\)'-dibromo-\(\alpha\)-xylenes by a method developed by Dittmer\textsuperscript{42}. The sulfinate precursor in our synthesis, 6,7-dichloro-1,4-dihydro-2,3-benzoxathiin 3-oxide (29), was synthesized in a 75% yield by the treatment of 4,5-dichloro-1,2-bis(bromomethyl)benzene\textsuperscript{43} (28) with sodium hydroxymethanesulfinate in DMF in the presence of tetrabutylammonium bromide. The \textit{in situ} generation of 4,5-dichloro-\(\alpha\)-quinodimethane, by the decomposition of 29, and the subsequent Diels-Alder reaction with ethyl (\(L\))-3-nitroacrylate (22) were conducted in benzene at reflux for 12 hours to give 6,7-dichloro-2-ethoxycarb-3-nitro-1,2,3,4-tetrahydronaphthalene (21, 75%) (Scheme 5).

It was expected that benzylic bromination of 21 with NBS followed by base-catalyzed dehydrobromination would result in aromatization due to the acidity of the protons at the C-2 and C-3 positions. However, only a 40% yield of ethyl 6,7-dichloro-3-nitro-2-naphthoate (20) was isolated.
Subsequent studies on this reaction provided a set of optimized reaction conditions. Under these optimum reaction conditions, reaction vessel jacket cooled with ice water, a tungsten light used as a radical initiator and extra NBS used, 20 was obtained in a 80-90% yield. This improvement not only increased the yield but also resulted in a much easier purification due to a reduction in the side-products.

The conversion of 20 to 6,7-dichloronaphtho[2,3-c]imidazol-2-one (IV) was completed in a three-step sequence (Scheme 6). Compound 20 was first reduced to afford ethyl 2-amino-6,7-dichloro-2-naphthoate (32). Hydrolysis of 32 gave 2-amino-6,7-dichloro-3-naphthoic acid (33) in almost quantitative yield. A reversal of this two step sequence caused some difficulties in the work-up. In the third step, 33 underwent a Curtius rearrangement followed by a subsequent addition-ring-closure\(^{44,45}\), under the treatment of diphenyl phosphoryl azide and triethyl amine in benzene at reflux, to give 6,7-dichloronaphtho[2,3-c]imidazol-2-one (IV) in 80% yield.

However, we found that the glycosylation of IV was very difficult. A Vorbruggen type glycosylation\(^{46,47}\) which has been successfully used for the glycosylation of various heterocycles failed under different solvent systems (CH\(_3\)CN or CH\(_3\)CN/CH\(_2\)Cl) and temperatures (25°C or 50°C). The sodium salt method, using 2,3,5-tri-O-acetyl-D-ribofuranosyl chloride\(^{48}\) as a sugar donor, also failed in our hands. We assumed that the electron-withdrawing 2-keto group of IV could be the reason for the unsuccessful glycosylation and this prompted us to investigate the preparation and use of 2-amino-6,7-dichloronaphtho[2,3-c]imidazole (V) as the heterocyclic intermediate.
The conversion of 20 to 2-amino-6,7-dichloronaphtho[2,3-β]imidazole (V) was accomplished by a conventional four-step reaction sequence (Scheme 7). First, 20 was hydrolyzed to give 6,7-dichloro-3-nitro-2-naphthoic acid (34) in almost quantitative yield. Under the treatment of diphenyl phosphoryl azide and triethylamine, 34 was converted to 2-amino-6,7-dichloro-3-nitronaphthalene (35) through a Curtius reaction. The reduction of 35 gave 2,3-diamino-6,7-dichloronaphthalene (14) in quantitative yield. Treatment of 14 with cyanogen bromide gave 2-amino-6,7-dichloronaphtho[2,3-β]imidazole (V) in quantitative yield.

For the glycosylation of V, several different methods were tried. No products were obtained with the Vorbruggen method under a variety of conditions. The fusion reaction of V with 1-0-acetyl-2,3,5-tri-O-benzoyl-b-D-ribofuranose at 180°C under vacuum also failed. Phase transfer glycosylation with 2,3,5-tri-O-benzyl-D-ribofuranosyl chloride in dichloromethane and 50% sodium hydroxide in the presence of Aliquat 336 gave multiple spots which indicated possible decomposition.
After several unsuccessful attempts to glycosylate V, we elected to study the preparation and use of 6,7-dichloro-2-methylmercaptanaphtho[2,3-d]imidazole (VI).

Scheme 8

NH$_3$, R=Bz
MeOH $\rightarrow$ $37$, R=Bz

NH$_3$, MeOH $\rightarrow$ $38$, R=H
Compound 14 was converted to 6,7-dichloronaphtho[2,3-d]imidazol-2-thione (36) by a cyclization with thiocarbonyl diimidazole. Compound 36 was then methylated to give 6,7-dichloro-2-methylmercaptanonaphtho[4,5-d]imidazole (VI) in an overall 64% yield. A small amount of the dimethylated product was also isolated from the methylation reaction. This yield was later improved to 81% by the treatment of 36 with methyl iodide in DMF without using potassium carbonate. Under these reaction conditions, the amount of dimethylated side-product was dramatically reduced.

Compound VI was silylated with BSA in dry 1,2-dichloroethane at room temperature for 30 minutes and this was followed by the addition of 1-O-acetyl-2,3,5-tri-O-benzoyl-d-ribofuranose and trimethylsilyl trifluoromethanesulfonate (TMSOTf) to give 6,7-dichloro-2-methylmercapto-1-(2,3,5-tri-O-benzoyl-d-ribofuranosyl)naphtho[2,3-d]imidazole (37) in a 97% yield. Compound 37 was deprotected using a saturated methanolic ammonia solution at room temperature to give 6,7-dichloro-2-methylmercapto-1-(d-ribofuranosyl)naphtho[2,3-d]imidazole (38) in a 96% yield. The b-configuration at the anomeric position of 38 was confirmed by a NOE difference experiment.

When 38, in methanol under -10°C was treated with 2.5 equivalents of a Cl₂ solution (chlorine gas bubbled into dry carbon tetrachloride), two nucleosides of approximately equal amounts were obtained as indicated by ¹H-NMR and HPLC. One of the compounds was subsequently assigned as 6,7,2,6,7-trichloro-1-(d-ribofuranosyl)naphtho[2,3-d]imidazole (I). The other compound was possibly a tetrachloro-nucleoside since only three aromatic peaks, with the absence of a peak for the methylthio group, were observed in the ¹H-NMR spectrum. However, when the reaction was conducted under -78°C for one hour with one equivalent of chlorine, compound 1 was generated exclusively.
The synthesis of 2-benzylmercapto-6,7-dichloro-1-(b-D-ribofuranosyl)naphtho[2,3-^d]imidazole (III) was accomplished from 6,7-dichloronaphtho[2,3-c]imidazo[2-thione (36) (Scheme 9). Compound 36 was treated with benzyl bromide in DMF to give 2-benzylthio-6,7-dichloronaphtho[2,3-^d]imidazole (39) in 90% yield. Compound 39 was then ribosylated by the Vorbruggen method to give 2-benzylthio-6,7-dichloro-1-(2,3,5-tri-O-benzoyl-b-D-ribofuranosyl)naphtho[2,3-^d]imidazole (40) in a 63% yield. Deprotection of 40 using saturated methanolic ammonia gave III in a 61% yield.

\[
\text{Scheme 9}
\]

The 6-benzo analog of DRB was synthesized by the treatment of 38 with W-4 Raney nickel to give 6,7-dichloro-1-(b-D-ribofuranosyl)naphtho[2,3-^d]imidazole (II) in a 52% yield.
These successful synthetic efforts have provided all of the proposed tricyclic dimensional probes (I, II, III). All of these dimensional probes have been evaluated for their antiviral activity against human cytomegalovirus (HCMV) infections in a plaque reduction assay in human foreskin fibroblast (HFF) cells.

Acknowledgment: This research was supported by the National Cooperative Drug Discovery Group for Infectious Diseases, the National Institute of Allergy and Infectious Diseases and the National Institute of Health (U19-AI31718). We should also like to thank Marina Savic for the preparation of this manuscript.
REFERENCES


[22] Underwood, M. R.; Stanat, S. C.; Townsend, L. B.; Drach, J. C.; Biron, K. K. "Inhibition of HCMV DNA Processing by a New Class of Anti-HCMV Compounds (Benzimidazole Ribosides) is Mediated Through the UL89 Gene Product", 8th International Conference on Antiviral Research, Santa Fe, NM, April (1995).


IN VITRO TESTING PROCEDURES FOR
LOCALLY APPLIED/LOCALLY ACTING PRODUCTS -
SUBSTITUTE FOR BIOAVAILABILITY/BIOEQUIVALENCE STUDIES?

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ABSTRACT

Locally applied/locally acting products are products that are applied locally and are intended to have their effect at the application site. Any systemic action is an undesired reaction. The active ingredient(s) of locally applied/locally acting products penetrate(s) the skin after release from the formulation and cause(s) a pharmaceutical response. The efficacy and/or safety of these products depends on the active ingredient(s) and the excipient(s). Every change in the components and composition could alter the extent of the penetration of the drug substance. The regulatory requirements are defined in the "note for guidance on clinical requirements for locally applied, locally acting products containing known constituents" and in the draft of the SUPAC-SS Guidance. In vitro release tests in the pharmacopoeias are performed for tablets, vaginal preparations, rectal preparations and transdermal patches. For locally applied/locally acting products no detectable blood levels are expected. Therefore, the performance of in vitro tests leads to results that cannot predict bioavailability or bioequivalence. With sufficient characterization of the test performance of the in vitro testing procedure for locally applied/locally acting products, these tests can be used for batch release purposes. Changes with regard to the SUPAC-SS Guidance can be monitored.

Keywords: Locally applied/locally acting; in vitro release; SUPAC-SS
INTRODUCTION

Locally applied/locally acting products are products that are applied locally and are intended to have their effect at the application site. Any systemic action is an undesired reaction. Various drug products represent topical dosage forms with local application: dermatological products such as creams, gels and ointments, or liquids and inhalatory products such as powders or aerosols for inhalation as well as products that are applied on mucous membranes such as eye preparations, ear preparations, nasal preparations, rectal preparations and vaginal preparations and even some oral preparations [1].

The efficacy and/or safety of these products depends on the active ingredient(s) and the excipient(s). In conclusion, a change in the formulation or in the dosage form may influence the efficacy and/or safety of the drug product. Every change in the components and composition could alter the extent of penetration of the drug substance and thereby the undesired side effects. Therefore, none of these topical dosage forms can, in essence, be considered similar [2].

Regulatory requirements:

The biopharmaceutical behaviour of topical drugs differs from that of oral drugs [3]:

![Diagram](image)

Therefore, the regulatory requirements are different to those for drugs with systemic action.

The "note for guidance on clinical requirements for locally applied, locally acting products containing known constituents" (III/3664/92-EN, Draft 6) defines the requirements for these drug products for a clinical dossier (Table 1). In order to demonstrate therapeutic equivalence,
clinical trials are usually necessary. Nevertheless, other models such as in vitro studies can be performed if they are adequately validated. For locally applied/locally acting products bioequivalence tests are, in general, not suitable for showing therapeutic equivalence as plasma levels do not reach detectable values although they may be important for safety reasons. [2]

The draft of the SUPAC-SS Guidance (Scale-up and Post Approval changes for Semi-Solid Drug Products) describes the performance of in vitro release tests as test documentation for most of the changes with regard to different strength from the bioavailability/ bioequivalence study strength, manufacturing site changes, changes in the batch size (10 times greater than the biobatch), changes in the manufacturing equipment or manufacturing process (both in the case of level 2 changes) or changes in components and composition (level 2 and level 3 changes) [4].

In Vitro Release Tests in the Pharmacopoeias

In vitro release tests for tablets are performed with regard to the different categories in the form of disintegration and dissolution with paddle, basket or flow-through cell apparatus (Table 2). Limits for the tests are laid down in the pharmacopoeias. Vaginal preparations are tested with regard to disintegration according to the requirements of the pharmacopoeias (Table 3). For rectal preparations testing procedures similar to those for vaginal preparations are laid down in the pharmacopoeias. Furthermore, the dissolution of suppositories is tested with a flow-through cell apparatus (Table 4). For dermatological preparations, a dissolution test for transdermal patches is performed (Table 5). A modified paddle apparatus is used for this purpose [4].

Other In Vitro Release Tests Applicable to Locally Applied/Locally Acting Products

Although a testing procedure for creams, ointments, and gels is not required in the pharmacopoeias, the permeation of the active ingredient(s) of locally applied/locally acting products through skin or artificial membranes can be tested with the Franz diffusion cell
The results of these in vitro tests are expressed in the amount permeated per area. A characterization of the test performance should include a validation of the analytical procedure and a development of the method for the testing procedure. This development of the testing conditions should include solubility and stability studies of the active ingredient, a selection of the (synthetic) support membrane, evaluation of the effect of the amount of formulation applied to the membrane and the effect of occlusion on the permeation. The (synthetic) support membrane must provide an inert support for the formulation allowing the drug to be released into the acceptor medium without causing any resistance to the diffusion of the drug from the formulation. Occlusion can have a significant effect on the release kinetics of a semisolid formulation. In consequence, occlusion could result in an increase in the humidity and temperature within the diffusion cell cap causing an accelerated dissolution of the active ingredient in the formulation [7].

CONCLUSION

Applicability of in vitro tests for bioavailability/bioequivalence studies of locally applied/locally acting products:

All the above-mentioned testing procedures can be performed for products that are resorbed and result in detectable blood levels. The in vitro and in vivo results can be compared with known mathematical operations. The specifications and testing conditions for the in vitro testing procedures are based on in vivo data. For locally applied/locally acting products no detectable blood levels are expected. As bioavailability defines the rate and extent at which the active ingredient(s) reach(es) the systemic circulation and as bioequivalence is a comparison of products that display the same bioavailability (i.e. identical rate and extent of absorption), the performance of in vitro tests of locally applied/locally acting products leads to results that cannot predict bioavailability or bioequivalence. With sufficient characterization of the test performance of the in vitro testing procedure, these tests can be used for batch release purposes. Changes with regard to the SUPAC-SS Guidance can be monitored.
REFERENCES

[1] Möller, H., Wirbizki, E., Loos, P., "In Vitro Characterisation of Other Preparations (e.g. Oral, Rectal, Vaginal)". Symposium on Quality and Interchangeability of Topical Products for Local Action (2nd EUFEPS Nuremberg Conference), December 1995


[6] The Hanson Vertical Diffusion Cell, Hanson Research (9810 Varial Avenue, Chatsworth, CA 91311, USA)

### Table 1  Requirements for the clinical dossier on locally applied/locally acting products

<table>
<thead>
<tr>
<th>I  Known active ingredient, not used locally before</th>
<th>Full dossier or appropriate bridging studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>II Abridged/hybrid studies</td>
<td>Relevant clinical studies</td>
</tr>
<tr>
<td>a Different indication</td>
<td>Relevant clinical studies</td>
</tr>
<tr>
<td>b Different dosing schedule</td>
<td>If possible, pharmacodynamic studies or local</td>
</tr>
<tr>
<td>c Different strength but usual dosing schedule</td>
<td>availability studies; possibly in vitro studies (e.g. eye</td>
</tr>
<tr>
<td>d Different formulation (e.g. cream → ointment; aerosol → powder for</td>
<td>drops) or argumentation in case of minor</td>
</tr>
<tr>
<td>inhalation)</td>
<td>differences. Otherwise clinical studies. Any safety</td>
</tr>
<tr>
<td>e Generic products</td>
<td>issue has to be addressed appropriately.</td>
</tr>
<tr>
<td></td>
<td>Clinical studies to demonstrate efficacy/safety</td>
</tr>
<tr>
<td></td>
<td>and/or if possible pharmacodynamic studies; if</td>
</tr>
<tr>
<td></td>
<td>necessary, studies of absorption, penetration and</td>
</tr>
<tr>
<td></td>
<td>local tolerance. See II c</td>
</tr>
<tr>
<td>III Type II variations</td>
<td>See II c</td>
</tr>
<tr>
<td>a Change in active ingredient(s) with regard to specification of the physical properties</td>
<td></td>
</tr>
<tr>
<td>b Change in the active ingredient(s)</td>
<td>See II c</td>
</tr>
<tr>
<td>c Change in application with regard to application device (e.g. inhalation chamber)</td>
<td>See II c</td>
</tr>
</tbody>
</table>
### Table 2  In Vitro Release Tests for Tablets

<table>
<thead>
<tr>
<th>Categories</th>
<th>Disintegration</th>
<th>Dissolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncoated tablets</td>
<td>Disintegration of tablets and capsules (2.9.1.)</td>
<td>≥ 75% in 45 min (water or 0.1N HCl at 37°C) (USP 23, Preface, Preamble)</td>
</tr>
<tr>
<td></td>
<td>≤ 15 min (water at 37°C)</td>
<td></td>
</tr>
<tr>
<td>Coated tablets</td>
<td>Disintegration of tablets and capsules (2.9.1.)</td>
<td>≥ 75% in 45 min (water or 0.1N HCl at 37°C) (USP 23, Preface, Preamble)</td>
</tr>
<tr>
<td></td>
<td>≤ 60 min (water at 37°C) or</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≤ 30 min (water at 37°C) for film-coated tablets</td>
<td></td>
</tr>
<tr>
<td>Effervescent tablets</td>
<td>≤ 15 min (water at 15°C to 25°C)</td>
<td></td>
</tr>
<tr>
<td>Soluble tablets</td>
<td>Disintegration of tablets and capsules (2.9.1.)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>≤ 3 min (water at 15°C to 25°C)</td>
<td>-</td>
</tr>
<tr>
<td>Dispersible tablets</td>
<td>Disintegration of tablets and capsules (2.9.1.)</td>
<td>May be applicable</td>
</tr>
<tr>
<td></td>
<td>≤ 3 min (water at 15°C to 25°C)</td>
<td></td>
</tr>
<tr>
<td>Gastro-resistant tablets</td>
<td>Disintegration of tablets and capsules (2.9.1.)</td>
<td>≥ 2h (0.1M HCl at 37°C)</td>
</tr>
<tr>
<td></td>
<td>≥ 2h (0.1M HCl at 37°C)</td>
<td>≥ 75% in 45 min (phosphate buffer solution pH 6.8 R at 37°C) (USP 23, Preface, Preamble)</td>
</tr>
<tr>
<td></td>
<td>≤ 60 min (phosphate buffer solution pH 6.8 R at 37°C)</td>
<td></td>
</tr>
<tr>
<td>Modified-release tablets</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tablets for use in the mouth</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
### Table 3 In Vitro Release Tests for Vaginal Preparations

<table>
<thead>
<tr>
<th>Categories</th>
<th>Disintegration</th>
<th>Dissolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moulded pessaries</td>
<td>Disintegration of suppositories and pessaries (2.9.2. ≤ 60 min (water at 37°C) ¹)</td>
<td>Dissolution test for solid dosage forms (2.9.3) ²)</td>
</tr>
<tr>
<td>Vaginal tablets</td>
<td>Disintegration of suppositories and pessaries (2.9.2. ≤ 30 min (water at 37°C) ¹)</td>
<td>Dissolution test for solid dosage forms (2.9.3) ²)</td>
</tr>
<tr>
<td>Vaginal capsules</td>
<td>Disintegration of suppositories and pessaries (2.9.2. ≤ 30 min (water at 37°C) ¹)</td>
<td>Dissolution test for solid dosage forms (2.9.3) ²)</td>
</tr>
<tr>
<td>Vaginal foams</td>
<td>-</td>
<td>?</td>
</tr>
<tr>
<td>Vaginal tampons</td>
<td>-</td>
<td>?</td>
</tr>
</tbody>
</table>

¹) Unless otherwise justified and authorised
²) Limits and test conditions based on in vivo data
Table 4  In Vitro Release Tests for Rectal Preparations

<table>
<thead>
<tr>
<th>Categories</th>
<th>Disintegration</th>
<th>Dissolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suppositories</td>
<td>Disintegration of suppositories and pessaries (2.9.2.)</td>
<td>Dissolution test for suppositories and soft capsules (2.9.3.)</td>
</tr>
<tr>
<td></td>
<td>lipophilic: ≤ 30 min (water at 37°C)  (^1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>hydrophilic: ≤ 60 min (water at 37°C)  (^1)</td>
<td></td>
</tr>
<tr>
<td>Rectal capsules</td>
<td>Disintegration of suppositories and pessaries (2.9.2.)</td>
<td>Dissolution test for suppositories and soft capsules (2.9.3.)</td>
</tr>
<tr>
<td></td>
<td>≤ 30 min (water at 37°C)  (^1)</td>
<td></td>
</tr>
<tr>
<td>Rectal solutions and suspensions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Powders and tablets for</td>
<td>Tablets: ≤ 3 min (water at 15-25°C)</td>
<td></td>
</tr>
<tr>
<td>rectal solutions and suspensions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Semi-solid rectal preparations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rectal foams</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rectal tampons</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Unless otherwise justified and authorised
Table 5  In Vitro Release Tests for Transdermal Patches

<table>
<thead>
<tr>
<th>Categories</th>
<th>Dissolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transdermal patches</td>
<td>Disk assembly method (2.9.4.)</td>
</tr>
<tr>
<td></td>
<td>Cell method (2.9.4.)</td>
</tr>
<tr>
<td></td>
<td>Rotating cylinder method (2.9.4.)</td>
</tr>
</tbody>
</table>

Figure 1  In vitro release of locally applied/locally acting products - testing apparatus [6]
REGULATORY REQUIREMENTS OF NOVEL DOSAGE FORMS

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ABSTRACT

For the registration of drugs incorporated in novel dosage forms a number of dedicated guidelines have been published in the areas of chemistry/pharmacy, toxicology, clinic, and pharmaco-economy. In this overview emphasis has been placed on those guidelines referring to in-vitro drug release and on the establishment of in-vivo/in-vitro correlations, on the toxicological consequences of the use of non-established excipients, and on the biopharmaceutical and therapeutical validation of extended-release preparations for well-known drug substances. Finally pharmaco-economical guidelines are considered, since the often higher cost of goods for the new delivery systems should be justified.

Keywords: novel dosage form, extended-release, controlled-release, in-vivo/in-vitro correlation, pharmaco-economics
INTRODUCTION

In today's health care environment there is an increasing need to develop, register and market drugs with more advanced delivery systems and packaging [1, 2]. One important reason is that new classes of drugs have been introduced such as biopharmaceuticals e.g. 3,4, leading to demands to delivery systems and packaging totally differing from those used for conventional drugs. Another technology push is coming from our increasing knowledge about the (patho)physiology of the human and about materials science. Finally the increased economic pressure requiring more cost-efficient pharmaetherapeutics is exerting a sort of marketing pull mechanism on the development of new drug delivery systems. Advanced delivery systems therefore may form a means of adding value to drugs:

- for the patient (the new medication may increase the patient's quality of life, it may be more easy to take, may require fewer doses, is perhaps more aesthetic to the senses, and may be more customised to the patient's individual needs)
- for the doctor (the new medication may lead to more optimal disease therapy/prevention, higher patient compliance, and less demands on doctor/nurse assistance)
- for the payer (the new medication may lead to demonstrable benefits, such as lower mortality/morbidity and reduced costs, e.g. due to less hospitalisation, and faster return to work).

Also packaging may substantially improve the quality of the medication, as is exemplified by the packaging of ophthalmic solutions for drug delivery to the eye [5].

Parallel to all the new developments in drug delivery and packaging, slowly also the corresponding regulations and guidelines are emerging from such authorities as the European Medicines Evaluation Agency, the USA Food and Drug Administration and the Japanese Ministry of Health & Welfare. Harmonisation of the regulations takes place in the International Conference on Harmonization process.

Whereas in the pharmaceutical company it is the task of the Department of Pharmaceutics to develop an optimal drug / advanced delivery system combination, it is the task of the Regulatory Affairs Department (RAD) to advise on suitable Regulatory Strategies for the specific system under development. The RAD representative in the Project Team in charge of developing the new therapeutic system should be able to highlight the regulatory peculiarities
associated with these advanced delivery systems. This article highlights some of the important Quality, Safety and Efficacy regulations and guidelines relating to advanced delivery systems.

**NOMENCLATURE**

Whereas the general terms "new dosage form" and "advanced delivery system" cover virtually all kinds of non-conventional drug delivery systems, especially for oral drug delivery specific prepositions have been defined, reflecting the kinetics of drug release e.g. slow-release, extended-release and delayed-release (see Table). An extended-release dosage form is described as a product allowing a reduction in dosing frequency when compared to a conventional dosage form e.g. a solution or an immediate-release dosage form [6]. More or less as synonyms for extended-release the prepositions sustained-release and "retard" are in use. The term sustained-release reflects the fact, that from a dosage form not all drug is released immediately, but is released over an extended period of time. The fraction released immediately is often referred to as the "loading dose", implicating that this effect is obtained purposely. The "delayed-release" dosage form has no altered kinetics of drug release apart from the fact, that there is a delay in the moment of first drug input; an example is an enteric-coated tablet.

Other well-known terms are "controlled-release" and "targeted-release". A controlled-release system releases the drug at a predictable rate governed by the drug delivery system, independent of environmental conditions. Targeted-release relates to the release of drug to a specific organ or at the cellular level, ideally to specific receptors. According to ICH-guidelines [7] a drug product with a new dosage form can be distinguished by its different pharmaceutical product type from the existing product, containing the same drug substance, and approved by the pertinent regulatory authority. Such pharmaceutical product types include products of different administration routes, new specific functionality/delivery systems and different dosage forms meant for the same administration route.
Table: Terminology for subclasses of modified-release products [6]

<table>
<thead>
<tr>
<th>subclass</th>
<th>plasma concentration peak</th>
<th>elimination half-life</th>
</tr>
</thead>
<tbody>
<tr>
<td>slow-release</td>
<td>lower $C_{\text{max}}$, later $t_{\text{max}}$</td>
<td>$t_{1/2}$ unchanged</td>
</tr>
<tr>
<td>extended-release</td>
<td>lower $C_{\text{max}}$, later $t_{\text{max}}$</td>
<td>apparent $t_{1/2}$ appreciably longer than intrinsic $t_{1/2}$</td>
</tr>
<tr>
<td>delayed-release</td>
<td>$C_{\text{max}}$ unchanged, lag time</td>
<td>$t_{1/2}$ unchanged</td>
</tr>
</tbody>
</table>

**CHEMISTRY / PHARMACY**

Controlled-release dosage forms differentiate from conventional dosage forms in their control over the drug release, which is often extended over longer periods of time. It is important to recognise, that not all drugs benefit from prolonged release and that a clear rationale should be given for their development [8].

Regulatory guidance on in-vitro release specifications for long-acting delivery systems can be found in two recent guidelines [6, 9]. According to FIP Guidelines [9], dissolution profiles of extended-release preparations should be specified at least at 3 different time points. The first specification should be set after a testing interval corresponding to an amount dissolved of 20-30% of labelled drug substance, to control potential dose dumping. The second specification point should be set at around 50% release of drug substance, to define the dissolution pattern. The final specification point should be set at least at 80% release of drug substance, to control quantitative drug release. Where both upper and lower limits are specified at any time point, the difference between them should usually not exceed 20% of the labelled content of drug substance in the formulation, unless limits have been shown to provide reproducible and acceptable in-vivo performance. For major changes, that are likely to have a significant impact on formulation quality and performance an in-vivo bioequivalence study, demonstrating the equivalence of the altered and the original system, is recommended in addition to extensive dissolution profile testing.

The FDA guidance [6] is currently still in draft form and is indicated for use with solid oral dosage forms. Nevertheless it is believed that the principles described herein can be generally applied to a much broader range of controlled-release dosage forms. It provides a good
indication of FDA’s current thinking and expectations with regard to the setting of in-vitro release specifications.

The development of a suitable dissolution test and suitable dissolution test conditions is the major development pharmaceutics item for an extended-release dosage form. Once an acceptable dissolution testing method and acceptable dissolution conditions have been selected, these must be fixed before proceeding any further and any manipulation of dissolution conditions after this stage is not appropriate. It is an accepted scientific principle to establish the in-vitro release specifications on the performance of the bio/clinical batches. Ideally, the specifications should be established such that all batches that have dissolution profiles within the upper and lower limits of the specifications, are bioequivalent. Minimally, these batches should be bioequivalent to the batches used in the clinical trials or to an appropriate reference standard.

For extended-release dosage forms the dissolution profiles of at least 12 individual dosage units from each batch must be determined. There must be a suitable distribution of sampling points to define the profiles adequately, and the coefficient of variation for mean dissolution of a single batch should be less than 10%. It is stated, that inter-batch variability should not be a primary consideration in setting the specifications, which means that manufacturing processes should not give rise to large batch-to-batch variations in drug release.

The correlation between in-vivo and in-vitro release (IVIVC) plays an important role for controlled-release systems. Based on an established IVIVC, changes in manufacturing methods and sites might be justified simply by showing adequate in-vitro release, instead of having to demonstrate bioequivalence in an expensive comparative pharmacokinetic study. One should keep however in mind, that the IVIVC is the characteristic of a defined controlled-release system/drug combination, and that profoundly changing this system might mean that the established IVIVC is not valid anymore. In case no quantitative IVIVC has been established, the maximum suggested range at any dissolution time point specification is in general ± 10% of label claim deviation from the mean dissolution profile obtained from the bio/clinical batches.
TOXICOLOGY

Although new dosage forms quite often contain excipients, additives or enhancers not mentioned in pharmacopoeia, they nevertheless may be "approved" implicitly by being part of an already licensed product. It may be worthwhile to compare data sheets or SmPC's in compendia or to consult the List of Pharmaceutical Excipients as issued by the FDA. Nevertheless such excipients may need more thorough evaluation if they are used for a new route of drug delivery, give greater exposure, or have novel combinations. Excipients not used previously should be dealt with as New Chemical Entities and should be subjected to the same scrutiny as new drugs, often requiring lengthy testing for toxicity.

For the formulation of controlled-release systems quite often polymeric excipients are used. Well-known examples are poly(dimethylsiloxanes), ethylene vinylacetate copolymers, and biodegradable polymers such as the copolymers of lactic acid and/or glycolic acid (PLA/GA) and the polycyanoalkylacrylates [10]. In case of biodegradable polymers, it is important to know the fate of the polymeric molecules and their degradation products in the body; and where, when and what distribution is relevant to the proposed use. The distribution from the site of administration dictates the design of the toxicity testing.

Some adverse reactions of drugs formulated in advanced drug delivery systems are not directly related to the drug substance, but are specifically attributable to the nature of the dosage form or the formulation used. Examples are the allergic reactions to the solubiliser "Cremophor EL" in Taxol [11], and the severe skin irritations occurring with a clonidine transdermal system [12]. Systemic effects from essentially local therapies can be found for instance for eye drop formulations [5]. Ciliary toxicity has been found for enhancers used in nasal formulations [13]. Also in inhalation systems preservatives such as benzalkonium chloride have given rise to hypersensitivity reactions. The purity of new excipients is of extreme importance; lysophosphatidylethanolamine contaminants have been found to depress phagocytic activity in a dose-dependent manner [14]. Also the purity of polymeric materials with respect to catalysts, glidants, anti-oxidants, etc. should be subject to extensive quality control. Formulation, packaging and handling of proteins should be such as to avoid denaturation and aggregation. The formation of aggregated insulin in subcutaneous insulin pumps has been found to cause a 6-time increase in serum amyloid level compared to that in healthy volunteers, possibly increasing the risk of development of secondary systemic amyloidosis [15]. In their overview
of drug events related to dosage forms and delivery systems, Uchegbu and Florence [16] conclude, that there is a definite need to label all medicines with all formulation ingredients and to include this information in the drug data sheet. This should enable that, not only during development, but also as part of post-marketing surveillance, attention is given to potential adverse effects associated with the delivery system.

CLINIC

The EC supplementary guideline "Clinical testing of prolonged action forms with special reference to extended-release forms" of July 1990 [17], defines the studies to be conducted in man, which are specific to new extended-release forms containing recognised active and safe drug substances so as to ensure a more prolonged action than the conventional pharmaceutical already marketed. The extended-release form is only considered acceptable if simultaneously:

- the drug substance is regarded effective and safe,
- the drug substance does not necessitate the repetition of high concentrations in the body and/or daily "wash-out" periods, to produce and maintain full therapeutic activity,
- the drug substance has a dose-response relationship such that a high level of adverse reactions would follow from the use of an increased drug substance content of a conventional dose form and/or can produce the desirable clinical effect with a lower dose in an extended-release preparation.

For well-known drug substances reformulated into extended-release dosage forms the guideline [17] states that:

- it is inappropriate/unnecessary to repeat the toxicological/pharmaceutical/clinical tests to define the inherent properties of the drug substance
- it is necessary to investigate the properties and effects of the new delivery system by studies in man
  - to establish that the new form exhibits extended-release in-vivo
  - to verify that a prolonged therapeutic effect is achieved
  - to verify that the mode of administration makes effective and safe treatment possible
For new drug substances in extended-release forms the standard efficacy and safety tests applicable to all new medical products should be conducted; however also the recommendations in the note for guidance [17] apply fully. Extended-release forms of well-known drug substances should be validated both biopharmaceutically and therapeutically.

The biopharmaceutical validation of the extended-release form [17] is used to establish that the new form exhibits extended-release in vivo. It requires a single dose study in healthy subjects with the conventional dosage form as reference, and a study at steady-state in patients or in healthy volunteers. Attention should be given to the following items:

1. Rate of drug delivery
2. Extent of drug delivery (poor bioavailability may reduce the level of activity significantly or increase inter-subject fluctuations to an unacceptable level),
3. Reproducibility of in-vivo performance (a significant larger variation between subjects than within subjects may be unacceptable),
4. Special characteristics, such as diet (influence on gastric pH / dose-dumping), site of drug application (e.g. skin), identical in-vivo and/or in-vitro performance for a series of extended-release forms different in dose (product surface area and/or unit content).

The therapeutic validation of the extended-release form [17] is used to verify that a prolonged therapeutic effect is achieved and that the mode of administration makes effective and safe treatment possible. The therapeutic activity must be maintained for the entire dosage interval. The intensity of activity must always be sufficient to justify the claims with regard to the indication. The nature, extent and frequency of adverse reactions must be approximately equivalent or inferior to those of the conventional form. Any claim that the extended-release form improves compliance should be duly justified. Depending on the indication, the influence of age, renal or hepatic impairment should be analysed.

The therapeutic validation of the extended-release product for well-known drug substances [17] comprises:

1. Pharmacokinetic studies (single dose and/or steady-state studies; in rare cases clinical studies may be considered necessary),
2. therapeutic studies (single dose / multiple administration; the drug/extended-release combination should be compared with either the validated conventional form, a placebo, an other recognised drug substance, or a dissimilar validated extended-release form. Attention should be given to the overall effectiveness of the treatment, adverse reactions, and the place of the new treatment among those already available on the market).

3. specific studies related to safety, to reveal systemic adverse reactions or e.g. local irritation or sensitisation.

An example of organ targeted, site-specific drug delivery is the use of inhaled dosage forms for the treatment of asthma. Here, comparison of systemic blood levels will not necessarily equate to therapeutic equivalence, because the concentration at the site of action may not be measurable and systemic bioavailability may not run parallel to therapeutic or pharmacodynamic effects. Therefore, such a type of delivery poses special difficulties in the assessment of quality and bioequivalence. The standards set for determination of clinical equivalence of inhaled dosage forms, in particular for metered-dose inhalers, have recently been reviewed [18].

PHARMACO-ECONOMICS

Although medical benefit stays the primary determinant for drug use, pharmaco-economic considerations are becoming increasingly important. Presently only Australia has enacted guidelines into national regulations; they have to be fulfilled to receive reimbursements for a pharmaceutical product in Australia. Other countries that have developed Health Economic guidelines are Canada, Italy, Spain, the United Kingdom and the United States. Health economic standards are still under development and no "golden standards" exist as yet [19]. One of the main reasons for the rather dissimilar guidelines is that the guidelines are used for quite different purposes. Patients focus on the drug's ability to cure the disease and improve the quality of life, whereas payers want cost efficiency in the delivery of services, and regulators want to ensure that the drugs are safe and effective. Consequently totally different pharmaco-economic analysis may be used, such as cost-benefit analysis, cost-effectiveness analysis, cost-minimisation analysis and cost-utility analysis. Nevertheless, all guidelines encourage to discuss medical data in the light of effectiveness rather than in the light of efficacy. Efficacy (and
safety) data are collected under ideal conditions in randomised clinical trials. Only in the field it can be judged whether the medication does what it is intended to do for a defined population.

The cost effectiveness of advanced therapeutic systems can be assessed, using measures such as purchase costs, total treatment costs and economic value of improved therapeutic outcomes. These therapeutic outcomes must be determined in real life. Controlled-release systems can add economic value to drugs by simplifying drug regimens and by controlling drug input. Programmed or pulsatile drug release taking into account the body's day/night rhythm may be more advantageous for the patient. Targeted release directing drugs to the diseased organs or sites, thereby avoiding as much as possible other places, may lower safety problems and enhance the effectiveness of the treatment. In a recent survey, it was concluded, that many of the controlled release products lead to simpler regimens with enhanced compliance, and that the control of drug input prevented the occurrence of super- and sub therapeutic plasma drug concentrations, thereby leading to improvements in drug efficacy, in adverse effect profile and in quality of life [20]. Despite the higher drug purchase costs, the overall care costs were lower.
REFERENCES


ABSTRACT

Besides the active principle a tablet formulation for direct compression normally contains a filler, a binder, a disintegrant, a glidant and a lubricant. During the last two decades materials were developed having filler/binder and e. g. disintegrating properties facilitating the development and production of tablets.

Three different materials, Ludipress®, consisting of α-lactose monohydrate 93 %, soluble povidone 3.5 % and crosslinked povidone 3.5 %, Avicel PH 200®, a granulated microcrystalline cellulose and Cellactose®, a combination of α-lactose monohydrate 75 % and powdered cellulose 25 %, were characterized by their powder properties and investigated for their compression behaviour alone and in combination with ascorbic acid as an example for a high dosed and highly water soluble drug and paracetamol as a high dosed and sparingly water soluble one. The three multi purpose excipients showed similar compresional pressure/hardness profiles but while the disintegration times of Ludipress® and Avicel PH 200® tablets were low and nearly independent over a compresional pressure range from 50 to 250 MPa, Cellactose® exhibited a tremendous increase in disintegration time above 100 MPa.

In direct compression the main problem with low dosed drugs is the content uniformity. By the formation of interactive mixtures using glibenclamide as a model drug it was shown that the coefficient of variation of glibenclamide content of the tablets was in the range of 1 % indicating excellent content uniformity. Differences were found in the dissolution behaviour of Ludipress® and Cellactose® corresponding to the disintegration behaviour of the tablets.

Keywords: Multi purpose excipients, co-processed materials, direct compression, binding capacity, interactive mixtures, content uniformity
1. Direct compression: possibilities and limitations

Direct compression of tablets was developed in the 1960s (1) and has become an interesting alternative over the last three decades. Apart from the economic point of view and the simplicity of the process direct compression offers many advantages compared to techniques such as pre-compression or wet granulation in terms of an improved process reliability and product stability. Thermolabile substances and active ingredients undergoing hydrolysis in contact with water can be tableted without difficulties. Besides the active principle a tablet formulation for direct compression normally contains a filler, a binder, a disintegrant, a glidant and a lubricant. Instead of a dry-binder in combination with a filler a so called filler/binder can be used offering filling and binding properties. The other excipients are in general the same as used in formulations prepared by dry or wet granulation. Over the last two decades attempts have been made to develop tablet excipients having not only filler/binder but also disintegrating or even lubricating properties. These excipients are called "co-processed materials" (2) or "multi purpose excipients" (3). This article deals with three recently marketed multi purpose excipients.

The decision to develop a tablet formulation either by granulation or direct compression is mainly influenced by the amount and the properties of the active ingredients being incorporated into the tablet. This is demonstrated by table 1.

Table 1: Possibilities and limitations in direct compression of tablets by low (Digoxin), medium (Diazepam) and high (Amoxicillin * 3 H2O) dosed drugs

<table>
<thead>
<tr>
<th>Substances</th>
<th>Amount in mg/tabl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digoxin</td>
<td>0.1</td>
</tr>
<tr>
<td>Diazepam</td>
<td>--</td>
</tr>
<tr>
<td>Amoxicillin x 3 H2O</td>
<td>--</td>
</tr>
<tr>
<td>Filler / binder</td>
<td>93.9</td>
</tr>
<tr>
<td>Glidant</td>
<td>0.5</td>
</tr>
<tr>
<td>Disintegrant</td>
<td>5.0</td>
</tr>
<tr>
<td>Lubricant</td>
<td>0.5</td>
</tr>
<tr>
<td>Tablet weight</td>
<td>100.0</td>
</tr>
<tr>
<td>Content of active</td>
<td>0.1 %</td>
</tr>
</tbody>
</table>

The table shows the amount of each substance in milligrams per tablet.
The first example, a low dosed digoxin tablet, contains only 0.1% of active ingredient. The percentages of glidant, disintegrant and lubricant are within the usual range. The amount of filler/binder to fill up to a tablet weight of 100 mg is 93.9 mg representing quite a high value. The compressional properties of this formulation will mainly be influenced by the properties of the filler/binder. From that point of view one would not expect difficulties during compression. The problem will be the content uniformity of the active ingredient which requires quite often a special treatment like pre milling with a part of the excipients, spray drying or pre blending before going into direct compression. The second formulation containing 10 mg of diazepam offers best conditions for direct compression. The content of active ingredient is 10% and high enough to prevent problems connected with content uniformity while the amount of filler/binder with 84% will still dominate the compressional properties of the tableting mixture. These medium dosed drugs are preferred in direct compression. The third formulation containing 1147.76 mg of Amoxicillin trihydrate is a high dosed tablet which is limited by an acceptable tablet weight of about 1500 mg. Due to the fact that the percentage of glidant, disintegrant and lubricant is still the same the amount of filler/binder available for this formulation is only 262.24 mg and the content of active ingredient is more than 76%. This formulation will mainly be influenced by the compressional properties of the active ingredient and due to the fact that antibiotics are not directly compressible a granulation step has to be considered. The three examples show that the dosage of the active ingredients is the most critical factor in decision making for direct compression. Besides that the ability of the filler/binder or the multi purpose excipient to take up a certain amount of a poorly compressible active ingredient which is also called the binding capacity of an excipient will be the second dominating factor in direct compression.

2. Multi purpose excipients
Multi purpose excipients are products offering more than one property in a tablet formulation. Besides their filling and binding properties they act as glidants and often as disintegrants. The incorporation of a lubricant into a multi purpose excipient as proposed by Parrott (4) seems to be difficult due to the fact that the amount of lubricant required for a certain formulation depends not only on the nature of the active ingredient but also on its amount in a formulation. Therefore the advantages of multi purpose excipients are:
- they reduce the number of raw materials to be handled
- they fulfill more than one function in a tablet formulation
they facilitate the production process: only the active drug and the lubricant should be added,
they reduce costs for analysis of raw materials and save space in the storage area.
The basis of multi purpose excipients are filler/binders which are modified either by a special production process or by the addition of additives and a special treatment bringing the desired properties into the combination. Thus multi purpose excipients were developed on the basis of cellulose (2, 5, 6, 7, 8), calcium phosphate (2), and lactose (9, 10, 11, 12, 13, 14). In the present study three multi purpose excipients, listed in Table 2, were used.

Table 2: Trade names and compositions of the three multi purpose excipients under investigation

<table>
<thead>
<tr>
<th>Trade Name</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ludipress</td>
<td>93.0 % α-Lactose Monohydrate</td>
</tr>
<tr>
<td>BASF</td>
<td>3.5 % Povidone 30</td>
</tr>
<tr>
<td>Cellactose</td>
<td>3.5 % Crosslinked Povidone</td>
</tr>
<tr>
<td>Avicel PH 200</td>
<td>Agglomerated Microcrystalline Cellulose</td>
</tr>
<tr>
<td>FMC</td>
<td></td>
</tr>
<tr>
<td>Cellactose</td>
<td>75 % α-Lactose Monohydrate</td>
</tr>
<tr>
<td>Meggle</td>
<td>25 % Cellulose</td>
</tr>
</tbody>
</table>

Ludipress is a co-processed material made from 93 % α-lactose monohydrate, 3.5 % povidone 30 as a soluble binder and 3.5 % of crosslinked povidone acting as a disintegrant. Avicel PH 200 is a specially treated microcrystalline cellulose, while Cellactose is a combination of 75 % α-lactose monohydrate and 25 % powdered cellulose. The powder characteristics of the three materials are listed in Table 3.

Table 3: Powder characteristics of the three multi purpose excipients under investigation

<table>
<thead>
<tr>
<th></th>
<th>Ludipress</th>
<th>Avicel PH 200</th>
<th>Cellactose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Particle Diameter (μm)</td>
<td>205</td>
<td>225</td>
<td>230</td>
</tr>
<tr>
<td>Bulk Density (g/ml)</td>
<td>0.517</td>
<td>0.276</td>
<td>0.380</td>
</tr>
<tr>
<td>Tapped Density (g/ml)</td>
<td>0.618</td>
<td>0.359</td>
<td>0.462</td>
</tr>
<tr>
<td>Hausner-Ratio</td>
<td>1.196</td>
<td>1.299</td>
<td>1.214</td>
</tr>
<tr>
<td>Angle of Repose (°)</td>
<td>30.3</td>
<td>35.7</td>
<td>36.2</td>
</tr>
</tbody>
</table>
The mean particle diameter of the three materials is similar, the bulk density is low for Avicel PH 200 and rises with increasing lactose content via Cellactose to Ludipress. For the tapped density the same behaviour was found while the Hausner-Ratio shows the invers ranking. The angle of repose indicating the flow properties of the products is lowest for Ludipress and highest for Cellactose which could be explained by the secondary electron microphotographs presented in figure 1.

Figure 1: Secondary electron microphotographs of Ludipress (a), Avicel PH 200 (b) and Cellactose (c); the bar is for all 3 pictures 100 μm
Ludipress exhibits round shaped agglomerated particles while Cellactose shows more or less irregular ones combined with long cellulose fibres. The shape of the Avicel PH 200 particles is in between these two materials explaining also the flow behaviour.

3. Compression behaviour of multi purpose excipients

The aim of a multi purpose excipient is to show advantages over a simple physical mixture of the components or a filler/binder alone. Figure 2 presents the compaction pressure hardness profiles of three different batches of Ludipress in comparison to the physical mixture of the starting materials and to Tablettose. This figure clearly demonstrates that the specially designed multi purpose excipient Ludipress is superior over the physical mixture. In addition an agglomerated pure α-lactose monohydrate, marketed under the name Tablettose, and being specially designed as a filler/binder for direct compression is in between Ludipress and the physical mixture with respect to tablet hardness/compaction pressure relation.

![Figure 2: Compaction pressure/hardness profiles of 3 different batches of Ludipress in comparison to the physical mixture of the starting materials of Ludipess and Tablettose, a pure agglomerated α-lactose monohydrate](image)

In total 16 different batches of Ludipress were investigated. The compaction pressure/hardness profiles of all 16 batches lay in between the three batches shown in figure 2. These three batches were choosen as follows: One batch was taken from the upper specification limit of the
particle size distribution showing the highest mean particle diameter. This batch resulted in the lower curve of the three Ludipress batches in figure 2. A second batch was taken from the middle of the particle size range showing the mean curve of the three batches and the third one was taken from the lower limit of the particle size range specified for the product resulting in the highest compaction pressure/hardness profile. All the other 13 batches were in between that range, indicating that the production process of Ludipress is reproducible and leads to tablets of a specific hardness at a defined compaction pressure.

The comparison of Ludipress and the two other materials Cellactose and Avicel PH 200 is given in figure 3.

![Figure 3: Compaction pressure/hardness profiles of Ludipress, Cellactose and Avicel PH 200](image)

Cellactose and Ludipress show similar compaction behaviour resulting in a slightly higher hardness of Cellactose tablets. The behaviour of Avicel PH 200 is different. The hardness values reach a plateau at around 200 MPa compaction pressure indicating a beginning capping tendency. In the normal tableting range of 50 to 150 MPa all three materials exhibit good compaction properties. The situation becomes different when looking at the compaction pressure/disintegration profiles as shown in figure 4.
Here Avicel PH 200 shows excellent low values over the whole compaction pressure range. Ludipress is slightly higher but also nearly independent from compaction pressure and in an acceptable range. In contrast Cellactose shows a tremendous increase in disintegration time at compaction pressures above 100 MPa. This could be explained by the composition of the three materials. Avicel containing pure microcrystalline cellulose shows good disintegration properties in general. Ludipress, based on α-lactose monohydrate and containing 3.5% of crosslinked Povidone as a disintegrant shows acceptable disintegration times due to the presence of the disintegrant. In contrast Cellactose which is prepared from 75% α-lactose monohydrate and 25% of a powdered cellulose does not have enough disintegration power. At increasing compaction pressures particles are coming closely together resulting in a hindered water uptake by capillary forces and therefore in a prolonged disintegration time.

One important parameter to characterize the properties of a multi purpose excipient is the determination of the so called binding capacity. Binding capacity of a filler/binder or a multi
purpose excipient for direct compression is defined as the ability of the material to take up a certain amount of active principle resulting in a tablet according to the specifications fixed before. The binding capacity should be determined using poorly compressible high dosed drugs. In minimum two drugs should be used one of them being highly water soluble while the other should show a poor water solubility. In the present work ascorbic acid and paracetamol were used as model drugs. The binding capacity was determined by mixing increasing amounts of the two materials with each of the three multi purpose excipients under investigation. The tablets prepared from these mixtures at 50 kN compressional force should meet the following specifications:

- tablet hardness: ≥ 50 N
- disintegration time: ≤ 50 minutes
- friability: ≤ 1 %.

Based on these specifications the binding capacity was determined for ascorbic acid with 30 % for all three materials and with 20 % using paracetamol for Ludipress and Avicel PH 200. With Cellactose paracetamol tablets could not be prepared due to a prolonged disintegration time. The limiting factor was in most of the other cases the friability. Avicel shows in combination with ascorbic acid a prolonged disintegration time while paracetamol in combination with Ludipress gives hardness problems. It should be noticed that ascorbic acid and paracetamol were used as fine powders. Therefore the upper limit of the binding capacity was also influenced by the flow properties of the tableting mixture. When using a crystalline material the binding capacity is higher. With fine powders the binding capacity could be enhanced by the addition of small amounts of colloidal silicon dioxide.

4. Formation of interactive mixtures

Interactive mixtures are defined as powder mixes of two or more components in which small particles adhere onto coarse carriers by attraction forces. This concept was introduced in 1975 by Hershey (15) under the name "ordered mixing". He assumed that the surface of a carrier particle would be completely covered by a monolayer of the fine drug. He claimed that in a so called ordered mixture the homogeneity of the powder mix is higher compared to a random one. Later on Egermann (16, 17) was able to show that the homogeneity of an ordered mixture is not better compared to a random mix, due to the fact that there is no "order" in the mixture but an interaction between coarse and fine particles leading to a "pseudo random" or "interactive"
The advantage of an interactive mixture is the higher stability of the final powder mix compared to a random one. In direct compression of low dosed drugs interactive mixtures seem to be a useful tool to stabilize the system and to fulfill the requirements of content uniformity. Micronized glibenclamide was used as a model drug in combination with the three multi purpose excipients under investigation. For comparison reasons Karion Instant, a spray dried sorbitol, was included into the investigation because this material has a high binding capacity for fine particles (18, 19).

The general procedure to prepare interactive mixtures using micronized glibenclamide and excipients is shown in figure 5.

Figure 5: Scheme of the formation of an interactive mixture containing glibenclamide as the active ingredient and of its sampling and assay.
After a first premixing step by hand the mixture is sieved through a 500 μm sieve to desaggregate lumps of the micronized active ingredient. Afterwards the main mixing step in a turbula mixer is performed for 30 minutes followed by a second sieving step through a 500 μm sieve. The prolonged mixing time was chosen to make sure that all lumps of glibenclamide are destroyed and a more or less uniform coverage of the micronized material on the surface of the carrier is achieved. After mixing and sieving samples were drawn from the mixture in an amount of 125 mg corresponding to the final tablet weight. These samples were analyzed for assay and content uniformity. The remaining powder mix was treated on an air jet sieve using a 63 μm sieve. Micronized glibenclamide having are particle range between 1 and 5 microns can pass this sieve while the coarser excipients are retained. Only the adhering micronized glibenclamide particles will not be separated by this sieving process. A final assay of the residual material on the sieve will lead to the amount of adhered particles onto the different excipients.

By plotting the glibenclamide content after sieving against the glibenclamide content directly after blending without sieving graphs are obtained showing the adhering tendency of glibenclamide to the different carriers as shown in figure 6.

Figure 6: Adhesion of micronized glibenclamide to coarse carrier particles after mixing and subsequent air jet sieving.
The adhering tendency of glibenclamide is in the same range for Ludipress, Karion Instant and Avicel PH 200. In contrast Cellactose shows much higher values of adhering glibenclamide. This could be explained by the different structures of the materials. The SEM picture of Cellactose (fig. 7) shows large cavities within the loose structure of the agglomerate. Glibenclamide is able to fill up these cavities under the formation of secondary agglomerates. This behaviour of micronized glibenclamide in combination with Cellactose has a highly stabilizing effect on the mixture preventing dimixing tendencies. This is supported by the secondary electron microscopic picture of figure 7, showing the re-agglomeration of glibenclamide crystals in a large cavity of Cellactose after mixing and air jet sieving.

Based on these results investigations will performed to determine the content uniformity of glibenclamide tablets of different strength prepared from the interactive mixtures using Ludipress and Cellactose respectively as multi purpose excipients. The results are given in table 4.
Table 4: Glibenclamide content (mean and standard deviation) of 10 single tablets of different strengths prepared from interactive mixtures using Ludipress and Cellactose respectively.

<table>
<thead>
<tr>
<th>Excipient</th>
<th>Glibenclamide concentration (mg/tablet)</th>
<th>Coefficient of variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Declaration</td>
<td>Mean</td>
<td>Coefficient of variation (%)</td>
</tr>
<tr>
<td>Ludipress</td>
<td>1.75</td>
<td>1.83</td>
</tr>
<tr>
<td></td>
<td>3.50</td>
<td>3.68</td>
</tr>
<tr>
<td></td>
<td>5.00</td>
<td>4.96</td>
</tr>
<tr>
<td></td>
<td>10.00</td>
<td>10.02</td>
</tr>
<tr>
<td>Cellactose</td>
<td>1.75</td>
<td>1.83</td>
</tr>
<tr>
<td></td>
<td>3.50</td>
<td>3.48</td>
</tr>
<tr>
<td></td>
<td>5.00</td>
<td>5.13</td>
</tr>
<tr>
<td></td>
<td>10.00</td>
<td>10.11</td>
</tr>
</tbody>
</table>

All mean values of the glibenclamide concentrations are within the ± 5 % level of the declaration. The coefficient of valuation was in all cases in the range of 1 % and none of the single values was outside of 85 % and 115 % of the declaration. This is an excellent result indicating the high degree of stability of an interactive mixture containing small amounts of an active drug. These findings are valid for both, Ludipress and Cellactose. The picture changes when looking on the dissolution behaviour of tablets prepared at different compressional pressures. This is demonstrated by fig. 8.

Figure 8: Dissolution rate of 5 mg glibenclamide tablets prepared from interactive mixtures based on Ludipress and Cellactose by direct compression. Ludipress as an excipient is superior compared to Cellactose showing no dependence from compaction pressure applied.
The dissolution rate of glibenclamide from Ludipress based tablets compressed at 75.9, 105.8 and 177.5 MPa is similar. The curves showing a much faster dissolution of glibenclamide from these tablets compared to Cellactose. For Cellactose a similar dissolution behaviour was found for tablets compressed at 59.8 and 97.7 MPa respectively. A tremendous decrease in dissolution rate was observed when glibenclamide containing Cellactose tablets were compressed at 176.9 MPa. These results are in good agreement with the findings for the disintegration time of Cellactose tablets. At compressional pressures above 100 MPa the disintegration time of Cellactose tablets increased tremendously. Therefore Ludipress as a multi purpose excipient in glibenclamide containing tablets is superior over Cellactose.
REFERENCES


THE ROLE OF THE HEME OXYGENASE SYSTEM IN THE MOLECULAR DYNAMICS OF MAMMALIAN GASEOUS MONOXIDE CELL SIGNALING

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ABSTRACT

The heme molecule plays a pivotal role in generation and function of the gaseous heme ligands, carbon monoxide (CO) and nitric oxide (NO) which are now recognized as important cell signals in a variety of organ systems. In turn, the heme oxygenase [HO] system [EC 1.14.99.3] which catalyzes oxidation of heme to produce CO plays a central role in CO-based cell signaling, as well as regulation of the hemeoproteins, nitric oxide synthase and soluble guanylate cyclase. The microsomal HO system consists of two forms identified to date: the oxidative stress-inducible protein HO-1 [HSP32] and the constitutive isozyme HO-2. These proteins differ in tissue distribution, primary structure, and regulation. This review highlights the current information on molecular and biochemical properties of HO-1 and HO-2 and their potential role(s) in the molecular dynamics of gaseous second messengers.

KEY WORDS: heme oxygenase-1 and 2, CO and NO generation, heme degradation, regulation of NO activity, CO regulation of cGMP-dependent activities
INTRODUCTION AND OVERVIEW

Recently, the gaseous signals, carbon monoxide (CO) and nitric oxide (NO) have gained recognition as a new class of cellular messages with function in a wide variety of mammalian organ systems under both normal and pathophysiological conditions. In general, the mammalian monoxide cell signaling system consists of few distinct enzymes which include nitric oxide synthase (NOS), the generator of NO, heme oxygenase (HO), the generator of CO, and soluble guanylate cyclase (sGC); a molecular target molecule for the gaseous monoxides. The concerted action of these components can account for essentially all known monoxide-based signaling.

The biological significance of the CO and HO system, as well as that of NO relates to the heme molecule. Indeed, heme may be viewed as the molecular cornerstone of monoxide cell signaling, a role which stems from the fact that all three enzymes (sGC, NOS and HO) are associated with the heme molecule and cyclic GMP-dependent signaling is mediated through monoxide interaction with the tetrapyrrole. The heme molecule is synthesized in a multistep enzymatic process that begins with *-aminolevulinate synthase [ALAS] and ends with ferrochelatase. Heme is oxidatively cleaved by the HO system, to form biliverdin, CO and free iron. This system was only identified some two decades ago as a distinct microsomal entity sensitive to metal ions [1]. A second, distinct form of the enzyme was identified a decade ago [2], these proteins are referred to HO-1 and HO-2, respectively [3, 4]. The HO isozymes are derived from different gene products. HO-1 also known as heat shock protein (HSP)32 is exquisitely sensitive, not only to metals, but to all kinds of stimuli and agents that cause oxidative stress and pathological conditions [reviewed in 5, 6]. Indeed, there is no other enzyme reported to be as exquisitely sensitive to so many stimuli of such diverse nature. Regulation of the HO isoenzymes is very different. HO-2 is not induced by the factors that increase HO-1; in fact the only chemical inducers of HO-2 identified to date are adrenal glucocorticoids [Gcs] [7,8].
Through new insights derived from development achieved in the HO field we now recognize heme metabolism as a bountiful source of physiological mediators. That is, all products of HO activity are now suspected to possess unique biological activities, a finding which has led to re-evaluation of the traditional view of heme degradation as a purely catabolic process. CO, like NO, is implicated as a signal molecule for generation of cGMP in biological systems. Fe released by HO activity regulates genes including that of NO synthase [NOS]. Bilirubin which is formed when biliverdin is reduced by biliverdin reductase [BVR] [9, 10] is a potent antioxidant [11, 12]. In addition, biliverdin itself is biologically active and inhibits viral replication, such as Herpes virus and HIV-1 proliferation in vitro [13]. Aside from the unique biological properties of HO activity products, the catalytic activity of the system plays a central role in maintaining cellular heme homeostasis and hemoprotein levels [6, 14, 15]; among the many hemoproteins and heme activated enzymes are NOS and soluble guanylate cyclase [sGC]. It is also important to bear in mind that HO protects the cell from the deleterious effects of the heme molecule, which is the most effective promotor of lipid peroxidation and oxygen free radical formation [16].

Here we highlight recent findings on the biochemical and molecular properties of the HO system and how they relate to the regulation of activity of two gaseous heme ligands, NO and CO. As a number of extensive and eloquent reviews [e.g. 17-22] describing the functions of NO have been published, this presentation will not deal with this topic.

THE KINDRED NATURE OF THE CO AND NO GENERATING SYSTEMS

Examination of the CO and NO generating systems quickly reveals an uncanny similarity. Both the HO and NOS have constitutive and inducible forms. And, the inducible form of the synthase, iNOS, is responsive to certain of the stimuli that induce HO-1, such as bacterial endotoxins, cytokines and reactive oxygen intermediates [23, 24]. This is not to suggest complete identity between regulators of iNOS and HO-1. HO-1 is responsive to more
categories and types of stimuli and agents than any gene described to date [reviewed in 6]. Also, both HO-1 and iNOS can be induced in many organs and tissues, and their induction involves gene activation and denovo enzyme protein synthesis. Again, this is not to imply that these proteins are not expressed in tissues under normal conditions. For example, in the normal spleen HO-1 is the predominant form, a phenomenon which likely reflects a constant exposure to hemoglobin heme; HO-1 and iNOS are highly induced in inflammatory processes in macrophages and in inflammatory cells, neutrophils, polymorphonuclear, and mononuclear cells [18, 24, 25]. On the other hand, HO-2, which is prominently expressed in neurons and endothelial cells of the central nervous and cardiovascular systems, respectively, and such forms of NOS are similar in that both are regulated by the adrenal Gcs [7, 8]. Interestingly, Gcs have opposing effects on expression of the two proteins in the brain with oxygenase being upregulated and the synthase being downregulated [7].

CO and NO share affinity for the heme molecule and they resemble one another in mediation of those activities that are cGMP-related [discussed below]. However, these monoxides fundamentally differ in chemical nature. NO is a free radical [CNO] and can react with oxygen-derived radicals to further generate a number of toxic free radicals [26, 27] whereas CO is not a free radical. Thus, CO cannot produce tissue damage associated with inflammation, or cytotoxicity to invading organisms that is caused by free radical species; such activities are mediated by CNO [22, 28]. Indeed, regulation of immune response by HO-1 activity may relate to CO produced by HO activity modulating CNO production and activity [29].

REGULATION OF THE CELLULAR TARGET OF MONOXIDE FUNCTION BY HO

The soluble form of guanylate cycles, sGC, is a hemoprotein [30-32] which exists as a dimer of two similar, but not identical, peptides that binds two hemes per dimer. The seminal findings of Ignarro and coworkers demonstrated that the presence of the tetrapyrrole ring is required for catalytic activity of sGC [30], and there is much evidence to suspect that heme is involved
in the enzyme activity and binding of the gaseous heme ligand NO [30, 33-35] and CO [33]. However, there is a clear need for further study of certain aspects of sGC interaction and activation by porphyrin containing complexes. Nonetheless, the interaction of sGC with heme can be reasoned to involve, for the most part, hydrophobic interactions with porphyrin side chains, as is the case of heme interaction with the catalytic site known as the Aheme oxygenase signature@ [36] HO isozymes, rather than the coordination of the metalloporphyrin through the chelated Fe core with amino acid side chains, as is the case with NOS [37-39] and cytochrome P450s in which an iron thiolate complex is formed between heme and a cysteine residue. Otherwise, various porphyrin complexes, without a metal chelate, could not activate the enzyme, and Zn- and Mg-protoporphyrin (PP) could not inhibit the enzyme activity [30]. HO activity is inhibited by such nonphysiological metalloporphyrins, which compete with Fe-protoporphyrin for the hydrophobic heme pocket [36, 40, 41]; the pocket does not have specificity for the chelated metal; instead it exhibits specificity for the porphyrin side chains [41]. Propionic acids on pyrrole rings 3 and 4 are required for porphyrin activation of sGC [30].

Just as it is accepted that activation of sGC, i.e increases in its Vmax for conversion of GTP to cGMP, is caused by interaction of its heme moiety with ligands, it has been thought [42] that CO generated by HO activity binds to sGC heme. However, while activation of the cyclase by CO has been demonstrated in vitro [33, 43], it does so much less effectively than NO. Furthermore, CO stimulation of cGMP production and promotion of cGMP-dependent activities has been reported in various cell and tissue preparations [44-46]. It is notable, however, that activation of sGC by CO, has been questioned by Burstyn et al [34] who did not observe a significant activation of purified sGC from bovine lung by CO. Regardless of a lack of complete clarity regarding the general mechanism of CO activation of sGC, there is a substantial body of data supporting the existence of an association in the cell between the HO system activity and functions that are related to cGMP synthesis. In fact, HO-2 protein or transcripts colocalize with cGMP and/or sGC in cells in which NOS is nearly absent [47-51] supports the assertion that activation of sGC by CO may be mediated via HO activity. A
number of sites of colocalization have been identified in the central nervous system and include the cerebellar Purkinje neurons, the hippocampal pyramidal neurons, locus coeruleus, and the olfactory bulb, wherein NOS expression is minimal or is essentially undetectable, and HO-2 is prominently expressed. In such cell preparations, a direct relationship between HO activity and cGMP production has been observed [52]. Furthermore, Ingi and coworkers [52] have shown in an olfactory receptor neuron culture system, a direct relationship between \(^{14}\)CO production and endogenous cGMP concentration.

Other investigators such as Luo and Vincent have also questioned the possibility of activation of cGMP by CO in vivo. Based on their studies [53] in which addition of high doses [50 FM] of Zn-PP in microdialysate was found to decrease the basal cellular levels of cGMP in cerebellar cortex, it was concluded that neuropharmacological effects of metalloporphyrins may result from their direct inhibition of sGC rather than from CO production. Such inhibition was not observed with lower concentrations [10 FM] of Sn-PP or Zn-PP, or with high concentrations of Sn-PP. Based on intimate knowledge of the molecular properties of HO isozymes and the reported properties of sGC it is possible to arrive at an alternate interpretation of the results. since HO heme pocket is not occupied, metalloporphyrins, be it the substrate or the inhibitor, can readily gain access to the pocket, which lends the HO activity highly sensitive to inhibition by minute concentrations of metalloporphyrins. Concentrations of non-physiological metalloporphyrins as low as 0.25 FM inhibit HO-2 activity by 50% [54]. sGC has been, on the other hand, classified as a heme containing protein [32], which by definition implies that heme is an integral part of the enzyme and is already associated with the heme pocket of the enzyme. Thus, high concentrations of an exogenous metalloporphyrin would be required to compete out and displace the heme bound to the protein. It follows that high concentrations of metalloporphyrins are likely to inhibit sGC. A similar example may be obtained from work in the cardiovascular area where Ny and coworkers [55] using 100 FM Zn-PP have observed inhibition of receptor-mediated relaxation of the rat aorta in a manner distinct from HO inhibition. In this study, Zn-PP inhibited atrial natriuretic peptide-mediated
increase in cAMP and cGMP and supports the non-selective actions of elevated concentrations of metalloporphyrins in biological systems. Accordingly, it is suggested that when using metalloporphyrins to examine biological activity of CO generated by the HO system, low concentrations of the compounds should be used to discriminate between sGC and HO inhibitions.

INTERPLAY BETWEEN HO AND NOS ENZYME SYSTEMS

A link between regulation of HO activity and NO production by NOS is becoming increasingly apparent. In this regard, one central question that emerges is, why should there exist a reciprocity of regulation between the HO activity and NO generation? Aside from maintaining/supplementing the ability of the cell to generate cGMP, it is plausible that the answer may, in part, be related to the significant role that HO activity plays in cellular defense mechanisms. Aside from activation of the sGC, interaction of CNO free radical with the reactive group[s] of enzyme proteins under select conditions can lead to chemical modifications, which result in inactivation of the enzymatic activities; hemoproteins would not be exempt from such interactions. CNO and its metabolites can interact with a variety of cellular components, including DNA, -SH groups, aromatic amino acids; transition metals, such as iron in the heme molecule and iron-sulfur clusters [22, 26-28]. For instance, the propensity of NO to bind heme iron of cytochromes can lead to their inactivation. Considering the exceedingly slow rate of dissociation of NO from the heme molecule [56], reversal of NO-mediated activation of sGC via dissociation of the NO-heme complex does not seem a viable mechanism to halt cGMP generation. Indeed, the ability to rapidly reverse a reaction is essential for maintaining cellular homeostasis, particularly in those cells that do not regenerate, or are slowly regenerated, such as the neurons. Thus, it seems reasonable to suggest that the activity of CNO is kept in-check by other biological means, the HO system may fulfill this role. Observations of the regulation of NOS and HO systems point to the likelihood that a controlling mechanism for NO production is the HO system.

HO-1 induction could regulate NO production in a number of ways, several of which reflect
the hemoprotein nature of NOS [37-39]. These could include: 1.) accelerated degradation of the newly synthesized heme which, in turn, could impair de novo synthesis of the holo NOS since the active site of NOS requires two heme molecules [57]. 2.) NOS as a P450-type hemoprotein may serve as do other intact and denatured forms of cytochrome P450 as substrate for both HO-1 and HO-2 [58]. By this analogy, an increased HO activity could be predicted to accelerate the turnover rate of NOS. The concentration of cytochrome P450 and HO activity show a reciprocal relationship, essentially all stimuli that increase HO-1 activity cause a concomitant decrease in cytochrome P450 [reviewed in 5 and 6]. 3.) Interaction of CO produced by the HO system could bind to the heme prosthetic moiety of NOS and inactivate the enzyme; both the neuronal and the macrophage forms of NOS have been shown to bind CO [37, 38]. 4.) Free iron released in the course of heme metabolism could elicit changes in nuclear transcription of consequence to NOS production [59]. 5.) Predominance of HO isozymes relative to that of NOS [47] might favor competition of the HO system for the requisite NADPH cofactor. In this context, because reduction of biliverdin to bilirubin by BVR also utilizes NADPH [9, 10, 60], competition for electrons would further favor the heme degrading system; BVR has very rapid kinetics of reaction.

The activity of the oxygenase system could indirectly modulate the synthase activity by regulating the negative feedback regulation by CNO on NOS activity and expression [61, 62]. Griscavage et al [61] have reported on the inhibition of NOS mRNA expression by NO, and NO, as would be predicted from its affinity for heme, has been shown to inhibit the NOS activity [62]. Thus, HO system activity, by controlling heme availability for NOS production, would modulate the negative feed back regulation that the synthase activity product exerts on its own production. Such a mode of regulation may underlie the recent finding that the inhibitor of HO activity, Zn-PP [63], causes activation of NOS in the rabbit internal anal sphincter [64].

It must be considered that interplay of NO and CO-generating systems is bidirectional in nature. That is, just as HO may exert influence on NO production, so too NO may regulate
HO activity. NO has been shown both to inhibit HO activity and to activate HO activity. Willis et al. [29] have shown that in vitro addition of CNO donor sodium nitroprusside(SNP) inhibits HO activity, whereas Motterlini et al. [65] have shown that incubation of endothelial cells with SNP for 6 h increases HO activity. This apparent discrepancy may be explained as follows: HO-2, which has two cysteine residues [66], the 2-SH groups render this protein highly vulnerable to free radicals [67, 68] such as NO which can modify such residues. Also, by binding to heme, which is the substrate for the HO isozymes, NO would inhibit HO activity [by preventing O2 binding to heme, which is necessary for heme oxidation]. Furthermore, as a free radical NO could induce HO-1 expression. It is notable that Sn-PP, a free radical generator [69] has such a dual mechanism of action [70]. Thus, the experimental system, time-frame for data collection, and parameters used for the assessment of HO isozyme expression and activity measurement, could influence whether a stimulation or inhibition of HO activity by an NO donor was observed.

NO might exert effects on the heme synthesis pathway, as well as components of iron storage with subsequent effect on HO activity and CO production. Specifically, NO may modulate ALAS, the rate-limiting enzyme in heme biosynthesis, and the synthesis of ferritin, the iron storage protein. Interestingly, iron has an opposing effect on regulation of ALAS and HO-1 activity; ALAS is down-regulated and HO activity is upregulated [71]. Iron regulation of ALAS and ferritin mRNA, as well as transferrin (TfR), involves activity of posttranscriptionally-regulated iron-responsive elements (IREs) [72, 73] present in the 3' or 5' UTR of the mRNA. In the case of HO-1, iron regulation of gene expression may involve free radical generation whereas regulation of ALAS by the metal may involve effects on the enzymes translation by interacting with IREs in the 5' UTR through the iron regulatory protein [IRP-1]. When IRP-1 is fully assembled as a 4Fe-4S cluster, it is the cytoplasmic enzyme aconitase and does not bind to IREs, in its Fe deficient form IRP-1 binds tightly to IREs and represses ALAS activity. IRP binds to 3' UTR IRE of ferritin mRNA and also represses its translation. In turn, iron metabolism, can be influenced by CNO where regulation of IRE-binding activity and repression of ferritin synthesis may be involved [74]. Thus, an extended
effect of this regulation may be the induction of HO-1 by increased free iron made available by a decreased need for the metal for heme biosynthesis and the repression of ferritin synthesis.

Depending on the cell type, forms of NOS and/or HO isozymes expressed and the type of stimulus to which the cells are exposed, the aforementioned mechanisms or components thereof, may represent modulation of NO production by HO activity. For instance, neuronal NOS, which is both particulate and microsomal membrane-bound [75], and is coexpressed in some neurons with HO-2 [50], would be subject to HO-2 regulation in those neurons wherein HO-2 is prominently expressed. Conversely, via the mechanisms described above, CNO could modulate HO-2 activity through free radical attack on -SH groups of HO-2. On the other hand, in macrophages wherein NOS is present predominantly in the cytosolic fraction, and HO-1 is highly inducible, this form of the oxygenase would become the predominant modulator of NOS activity, and CNO would activate HO-1 gene expression. In an organ such as the testis, in which highest levels of HO-2 are present in germ cells and exceedingly low levels of BVR are present [10], competition for NADPH with BVR would not be a significant factor, but competition with HO-2 for the reducing equivalents would become formidable for NOS [if present]. Endothelial NOS is largely plasma membrane-bound [76], whereas HO isozymes are microsomal membrane-bound; thus, it is unlikely that HO isozymes directly degrade the synthase. From these examples it is reasonable to suggest that the dynamics of monoxide generation and interaction of its enzymatic components is inextricably linked to the physiological context being considered.
REFERENCES


[57] Xie, Q-W., Leung, M., Fuortes, M., et al., AClimaxing Analysis of Mutants of Nitric Oxide Synthase Reveals that the Active Site Requires Two Hemes@ Proc. Natl. Acad. Sci. USA, 93, 4891-96 (1996).


THE EMERGENCE OF PHARMACOEPIEMIOLOGY AS A DISCIPLINE

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ABSTRACT

Pharmacoepidemiology is a hybrid scientific discipline drawing upon the foundations of both pharmacology, the study of drugs; and epidemiology, the study of disease. These disciplines have been closely aligned since the first part of this century, when many pharmaceutical products emerged, and the occurrence and causation of many diseases was fully explored. The importance of a complete analysis of the outcomes of drug therapy, either positive or negative, has become a global and crucial concern as more and more drugs with a narrow therapeutic window have entered the international marketplace. Just as a new therapeutic agents must have a complete chemical, pharmacological, pharmacokinetic, and clinical profile before market entry; so must a complete examination of the outcomes (clinical, economic, humanistic) of the product after market entry proceed. In addition, drugs of abuse or social drugs (alcohol, tobacco, etc.) can be examined from a pharmacoepidemiologic standpoint so as to assess the negative effects of continued use. Pharmacoepidemiology encompasses the review of clinical trials, case control studies, cohort studies, voluntary adverse drug reaction and adverse drug effects, and postmarketing assessments. Pharmacoepidemiology holds great promise and potential to further aid in the assessment of outcomes of pharmaotherapeutic treatments. In order to reach its full potential, pharmacoepidemiology must assume a global, transcontinental focus which encompasses ready and facile communication across disciplines, linked databases; and provide for an enhanced communication of research findings.

Key Words: Therapeutic outcomes, pharmacology, epidemiology, pharmacoepidemiology
INTRODUCTION

The disciplines of pharmacology and epidemiology converge in a new discipline - pharmacoepidemiology. Pharmacoepidemiology encompasses the crucial examinations of the outcomes of drug therapies either positive or negative. These disciplines further intersect when iatrogenic disease causative factors are determined, for example vaginal adenocarcinoma caused by diethyl stilbesterol, and endometrial cancer caused by exogenous estrogen supplements[1]. The promise of pharmacoepidemiology is great, but the proof of its maturation and success lies in a maturing of the discipline that must encompass scientific rigor and solid, valid replication of designs, analyses, and communication of findings.

Epidemiology

Epidemiology can be defined as the study of disease, including exploration of the factors that lead to occurrence, long term outcomes, degree of morbidity over time, and methods and means of ameliorating the end results of various disease states. These factors may include vectors for the transmission of infectious diseases, or health, diet, or risk factors for the occurrence of disease. Epidemiology also includes the analysis of various types of cancers, causative agents, precipitators of occurrence, and the end results of its impact. Epidemiology also encompasses preventative aspects that include methods of prevention of the occurrence of disease, such as tobacco and smoking cessation as a method of reducing various cancer related risks.

Each occurrence of a hitherto unknown disease leads to a renewed appreciation of epidemiology and epidemiologic techniques. For example, the growing, global emergence of the AIDS epidemic has heightened the importance of epidemiology.

Pharmacoepidemiology
Pharmacoepidemiology can be seen as a subcomponent of epidemiological studies. The term pharmacoepidemiology and subsequent studies began approximately fifteen years ago. Since then, there has been an emergent realization of its importance globally. Before this time, there had been a focus on studies of this nature. However, recently the dissemination of pharmacoepidemiologic research, results, and findings has truly been exponential in nature from one end of the globe to another. The major impetus for the importance of pharmacoepidemiological studies rests with the crucial nature of the importance of the use of pharmacotherapy to treat various illness and symptoms. Accompanying this focus on the use of drugs is a need to examine the wide range of effects and consequences of the use of an ever increasing and potent array of pharmacologic agents.

Importance of Pharmacoepidemiology

As therapeutic drug use has increased, a more focused examination of the beneficial and adverse effects of drugs has heightened the importance of pharmacoepidemiology. A crucial need exists to monitor the outcomes of the use of drugs in health care delivery systems. Drug outcomes have the potential to be positive or negative. Positive outcomes include beneficial treatment effects, disease or symptom abatement, cure of infection, relief of pain, or prevention of untoward sequelae of the progression of various diseases or disease processes. Negative drug effects may include self-limiting adverse drug reactions (ADRs) or adverse drug effects (ADEs). Further negative drug effects may include lack of efficacy, ineffectiveness, or interactions with other drugs negating the effects of one or both, or synergistically impacting upon the effects of one or many other drugs. In addition, severe untoward effects may progress to irreversible states, anaphylaxis, or death. The importance of monitoring these drug therapy outcomes both positive and negative cannot be overstated.

The emerging importance of pharmacoepidemiology has been noted:

“current issues pertaining to drug therapies are no longer isolated in individual medical or pharmacy practices, sequestered to specific patient populations or subgroups of
patients, nor are they problems for governmental regulatory bodies only. They have become global concerns affecting a worldwide array of individuals [2]."

Focal interest in the discipline can be found in the following groups of interested observers:

- Researchers in the pharmaceutical industry, including those involved in designing and monitoring clinical trials, as well as those involved in post marketing surveillance of pharmaceuticals,
- Clinical practitioners involved in clinical drug trials or in assessing the outcomes of drug therapies,
- Academicians in various health professional schools,
- Researchers in schools of public health, epidemiologists, and other related professionals,
- Researchers in government drug regulatory agencies,
- Global multidisciplinary teams examining multicenter and international comparisons of the efficacy or lack thereof of drug products.

**Types of Pharmacoepidemiologic Studies**

Examples of pharmacoepidemiologic studies include:

- Controlled clinical trials to analyze effectiveness of drugs, (the comparison of a drug with placebo, gold standard therapy, or with a similar drug in the same class, or a drug in a different therapeutic class).
- Demographic studies to determine patterns of usage of drugs, (analyses to examine who is using what drug in multiple populations and subpopulations).
- Pharmacoeconomic analyses of selected pharmaceuticals, (analyses of cost-effectiveness, cost-benefit, cost-minimization, or cost utility, see Table 1 for comparison of these types of pharmacoeconomic comparisons).
- Observational epidemiological studies:
⇒ cross-sectional studies (examining components of drug use across populations at a particular point in time),
⇒ case control studies (matching subjects with others sharing similar characteristics),
⇒ cohort studies (using a sample of patients with a common characteristic, e.g., the same disease, same age group, and/or same drug therapy),
⇒ drug utilization surveys (examining patterns of usage in a sample or population of patients, assessments of the drug use process in general),
⇒ automated databases (using computer linked data sources to examine drug use events of note; technological aspects of postmarketing surveillance, including evaluation of computer facilitated linking of data bases and subsequent research),
⇒ spontaneous reports (prescription even monitoring (PEM) system in Great Britain, United States Food and Drug Administration spontaneous reporting program, the regional pharmacovigilance centers in France, and the worldwide medical literature),
⇒ innovative methods of adverse drug reaction reporting and solicitation (not limited to but including patient reports),
⇒ epidemiological assessment of patient compliance,
⇒ Various aspects of the assessment of the outcomes of drug therapies,
⇒ a. clinical,
⇒ b. economic (please see Table 1),
⇒ c. humanistic (quality of life, patient compliance, adverse drug effects, patient satisfaction with care).

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Selected Examples of Varying Types of Pharmacoepidemiologic Studies

In order to more fully gain an appreciation of pharmacoepidemiology and its application throughout the world, it may be useful to examine the application of various pharmacoepidemiologic techniques in varying study designs from literature sources throughout the world.

**Demographic studies.** In a study to determine progressive use of benzodiazepines after an initial prescription, Nuettel and Maxwell[3] determined, in a cohort study using data from 1979-1986 on benzodiazepine use derived from the Saskatchewan Drug Plan in Canada, that 23.5% of the sample went on to receive at least 3 prescriptions within any 5 month period. Repeat users showed a greater preponderance of elderly users than the new users who had the larger proportion of users at younger age groups. The proportions of persons going on to repeat use increased steadily with age from 8% for the 20-29 year age group to 39% for the greater than 80 year age group. It was concluded that it is clear that the first prescription does lead to more benzodiazepine prescriptions within a limited amount of time and that the probability of this occurring varies by age group.

In another study examining the use of contraindicated therapies, Fincham and Makoid [4] examined the concomitant use of nonsteroidal anti-inflammatory drugs (NSAIDs) and histamine two (H2) antagonists in a sample of 832 elderly retirees and dependents in a rural managed care setting. There were a total of 45 patients taking both classes of drugs on a long-term basis. The long-term consequences of such usage need further exploration and analysis.

**Observational epidemiological studies.** Blancquaert and Gray[5] examined the extent to which NSAIDs are used by children under 16 year of age. In order to determine the indications for their use, 1448 Quebec physicians were surveyed by mailed questionnaire to estimate approximately how often, and for which indications, NSAIDs had been prescribed over the past year for children under 16 year of age. The sample included all pediatricians and
rheumatologists in the province, and a stratified random sample of general practitioners. A response rate of 81.5% was achieved. The proportion of physicians prescribing NSAIDs varied with specialty and clinical condition. The highest proportions were recorded for dysmenorrhea and juvenile rheumatoid arthritis, followed by tendinitis/bursitis, musculoskeletal trauma, and recurrent headaches. It was concluded that NSAIDs are prescribed for conditions for which they are not currently recommended in the pediatric population.

Herings et al[6] evaluated the size of the population in the Netherlands potentially at risk for developing torsades de pointes while on terfenadine or astemizole therapy, the PHARMO drug data base was used to determine the prevalence of prescribing supratherapeutic doses of either drug or the concomitant exposure to terfenadine and cytochrome P-450 inhibitors. The prescribing of supratherapeutic doses and the concomitant use of terfenadine and cytochrome P-450 inhibitors was low. Sensitivity analysis showed that the risk of fatal torsades de pointes has to be as high as 1 in 10,000 to cause 1 death in the Netherlands in 1 year.

Ahonen and Enlund[7] performed a study to characterize analgesic users (including users of anti-inflammatory drugs) and the analysis of the relationship between analgesic use and occurrence of pain and psychoneurotic symptoms in a sample of Finnish farmers with the final sample of 12,056 respondents, ages 18 to 65 years. The use of prescribed analgesics was most strongly associated with the occurrence of pain, chronic morbidity, and frequent use of physician services, while occurrence of psychoneurotic symptoms and gender (female) were the best predictors of nonprescribed analgesic use.

Cross-sectional studies. Fincham[8] categorized a series of adverse drug reactions (ADRs) occurring in elderly patients which were reported to a statewide reporting program in Mississippi (U.S.A.) is described. Of the 497 ADRs reported to the program, a total of 122 (24.5%) occurred in patients over 60 year of age. A total of 80 (65.6%) of these reactions could be classified as allergic responses that were self-limiting.
Case control studies. Moride and Abenhaim[9] completed a case control study to determine if past experience with a drug may modify the risk of adverse events associated with current use of the drug. An empric, case-control study was conducted in 244 patients with upper GI bleeding (UGIB), age 68 year and over, and 615 matched controls. Data on all medications dispensed to the patients during the 3 year preceding admission were obtained. Recent use (within 30 days prior to admission) of non-aspirin NSAIDs increased the risk of UGIB. The estimate of relative risk (RR) was 3.4. Use of NSAIDs in the previous 3 years was associated with a lower risk of UGIB; the estimate of RR was 0.7. The estimate of RR for first-time users was 2.7 versus 3.0 for those who had used the drugs at least once in the past 3 years. It was concluded that these results provide empirical evidence of a depletion of susceptibles effect whereby patients who remain on the drugs are those who can tolerate them, while those who are susceptible select themselves out of the population at risk.

Ray et al[10] performed an analysis of data collected from Medicaid (U.S.A.) files of 1021 elderly patients with hip fractures and 5606 controls to assess the risk of hip fracture associated with current use of psychotropic drugs. Persons treated with hypnotics-anxiolytics having short elimination half-lives had no increased risk of hip fracture. By contrast, a significantly increased risk was associated with current use of hypnotics-anxiolytics having long elimination half-lives, tricyclic antidepressants, and antipsychotics. It was concluded that these data support the hypothesis that the sedative and autonomic effects of psychotropic drugs increase the risk of falling and fractures in elderly persons.

Cohort studies. Van Den Eeden and Friedman[11] conducted a postmarketing surveillance hypothesis-generating screening program of prescription drugs that examined 215 drugs and 56 cancer sites in a cohort of 143,574 Kaiser Permanente Medical Care Program members whose prescription records were computer stored between 1969 and 1973 and in whom cancer occurrence was determined through 1988. New findings were associations of indomethacin with stomach cancer, prednisone with myeloid leukemia and lymphosarcoma, sulfamethoxazole with lymphosarcoma, thiazides and isoniazid with gallbladder cancer.
Rawson[12] completed a study to assess the impact of data concerning preexisting serious gastrointestinal (GI) disorders before piroxicam and sulindac therapy in an acute adverse drug reaction alerting program. A cohort study of 20,000 patients dispensed piroxicam and 20,000 patients dispensed sulindac through the Saskatchewan drug plan was conducted. Rates of physician services for peptic ulceration or GI hemorrhage in the 30 days after starting piroxicam or sulindac therapy for patients who had services or hospitalizations for GI disorders in the 90 days before their prescriptions were significantly greater than corresponding rates for patients without a recent history of these conditions. Hospitalization rate of GI disorders in the 30 days after starting sulindac therapy for patients who had services for these conditions in the previous 90 days was significantly greater than the rate for patients who did not. It was concluded that data regarding preexisting health conditions are essential in adverse drug reaction alerting programs and in all evaluations of adverse reactions.

Drug utilization surveys. Banfi et al[13] examined the prevalence of epilepsy by collecting data from anticonvulsant prescriptions. A population of 988,828 people over 6 months in 1992 and over 6 months in 1993 was studied. The prevalence of the disease based upon the utilization pattern of defined daily doses was found to be 5.2 per 1000 inhabitants in 1992 and 4.9 per 1000 in 1993.

Johnson et al[14] developed a measure of average daily dose using automated outpatient prescription drug data from a large database, and examined the use of the measure to show if the average daily dose of alprazolam increased after approval for panic disorder. Applying the measure, the average daily dose of alprazolam increased after it was approved for use at higher doses, and no seizure disorders were observed among any users with highest average daily doses. It was concluded that average daily doses can be estimated from large automated prescription databases.
Automated databases. Andrade et al[15] conducted a study to evaluate the utility of clinical automated databases to facilitate the study of drug discontinuations using three methods. Overall, 75% of discontinuations flagged by the automated databases were confirmed in the medical charts. In new users, 97% of drugs identified as drug switches in the computerized files were confirmed as discontinuations by the medical charts. In the second method, 54% of members with greater than 6 months between the last refill for an antihyperlipidemic drug had stopped therapy according to the written records, and in the third method, 90% of those flagged with inactive or omit had discontinued therapy per the written records. This study demonstrates the utility of clinical automated databases to facilitate the study of drug discontinuations, reducing the cost and time required for the evaluation.

Farris et al[16] quantified the extent of missing data in the days supply field in computerized prescription claims (CPCs) and the validity of data that was provided was assessed using CPCs from a southeast Michigan (U.S.A.) health maintenance organization (HMO) for April 1990 to March 1991. Data analysis of all 228,876 records revealed that less than 0.3% of the records had missing data in the days supply field. Quantity dispensed and directions for use were abstracted from the original prescriptions at 3 pharmacies for 300 randomly selected prescriptions to calculate a prescription days supply. Sufficient quantifiable data were available for 82% of the sample. The computer and prescription days supply were statistically different, although 173 (70%) records were in exact agreement between computer and prescription days supply. It was concluded that these findings suggest that CPCs are suitable for drug use review purposes where population rather than individual trends are examined.

Spontaneous reports. In a study of aseptic meningitis following mumps vaccine reported to French regional pharmacovigilance centers, Jonville et al[17] completed a retrospective survey of 54 cases of meningitis reported to the regional pharmacovigilance centers or to the manufacturer from the time each vaccine was launched up until June 1992. Twenty cases were temporally associated with the administration of a monovalent mumps vaccine and 34 with a trivalent measles, mumps, and rubella vaccine (MMR). A mumps virus
was isolated in 4 cases in the cerebral spinal fluid (CSF) and an Urabe-like strain was characterized twice. A probable mumps origin was assumed in 17 other cases where the patients presented with other clinical or biological signs of mumps infection. The clinical outcome of meningitis, known in 87% of the population, was always favorable. It was concluded that even considering that the actual incidence of AM is much higher when assessed by active surveillance studies, the risk/benefit ratio of mumps vaccine remains in favor of vaccination.

Wild[18] has described the events and actions leading up to the worldwide withdrawal of terodiline hydrochloride (Micturin) from the market in 1991. It was noted that the drug was withdrawn after the discovery of an association with serious cardiac arrhythmias, notably torsades de pointes.

Innovative methods of adverse drug reaction reporting and solicitation. In an examination of adverse drug experience reports submitted by pharmacists and physicians to the FDA, Ahmad et al[19] examined the quality of information in adverse drug experience (ADE) reports submitted directly to the U.S. Food and Drug Administration (FDA) by pharmacists and physicians compared with manufacturer-channeled 15-day reports. 589 ADE reports with serious outcomes associated with 9 new molecular entities were evaluated. Results showed no substantial difference in a subjective assessment of the quality of information in the reports submitted by pharmacists or physicians, irrespective of whether these reports were submitted directly or via manufacturers. It was concluded that this study suggests that pharmacists, especially those in hospitals, submit high quality ADE reports to the FDA, and thereby play an important part in protecting public health by promptly reporting serious adverse events.

In an examination of spontaneous reports of drug-induced erythema multiforme, Stevens-Johnson syndrome and toxic epidermal necrolysis in Denmark, Gaist et al[20] reviewed all reports concerning these diseases submitted to the Danish Committee on adverse drug reactions during the period 1968-1991. Two hundred cases of erythema multiforme, 74 of
Stevens-Johnson syndrome, and 29 of toxic epidermal necrolysis were identified. One hundred and twenty-eight different drugs were reported as causal agents. Major drug groups involved were antibiotics, nonsteroidal anti-inflammatory agents, anticonvulsants, and analgesics. The reporting fraction for erythema multiforme and Stevens-Johnson syndrome were estimated to be from 10-30%, and for toxic epidermal necrolysis to be from 25-50%.

Montero et al [21] describe a European Union pilot study to evaluate the possibilities of telematic transmission of pharmacovigilance data between the national administrations in Spain, the United Kingdom, and France. The network uses international standards to transmit urgent drug alerts among the 3 countries.

Moore and colleagues[22] described the importance of communication in pharmacovigilance and its importance to drug information and drug safety. They stress the importance of telematics, electronic data exchange, regulatory processes, and transmission of information to the World Health Organization (WHO) and the European Community (EC) health authorities.

Neutel and Johnansen[23] describe the use of record-linkage in pharmacoepidemiology, including the process of linking records, quality of resulting data sets, advantages and disadvantages, ethical considerations, and possible future directions.

Various aspects of the assessment of the outcomes of drug therapies,

Clinical. Van Staa and Abenhaim[24] examined hypoglycemic patients in centralized data banks in the United Kingdom. Results showed that 90.7% of the discharge summaries for hypoglycemia were adequately recorded in the data bank, and 88.6% of the data bank codes indicating hypoglycemia hospitalizations were correct. For most of the other reviewed medical conditions, it was found that the sensitivity of computer notation and positive predictive value of computer codes was over 90%. The agreement between information on the severity of diabetes and general practitioners questionnaire information was
over 85%. It was concluded that the data in the VAMP research data bank have a high level of completeness and validity.

Chen et al[25] examined the elevation of cholesterol and triglyceride levels with concomitant oral contraceptive and isotretinoin therapy. A significant association between concomitant oral contraceptive use and hypertriglyceridemia and hypercholesterolemia among female isotretinoin users was demonstrated. An unusually high frequency of concomitant oral contraceptive use among both hypertriglyceridemia and hypercholesterolemia was observed. It was concluded that there may be an interactive effect between oral contraceptives and isotretinoin on the elevation of serum triglyceride or cholesterol levels.

Delargy and Li Wan Po[26] performed a meta analysis to try to determine if in fact terfenadine and astemizole are non-sedating antihistamines. A meta-analysis of 55 clinical trials (18 cross-over trials and 37 parallel group trials) was performed, using chlorpheniramine as a positive control. Results indicated that both astemizole and terfenadine were no more likely than placebo to induce drowsiness. In the cross-over trials, none of the 3 antihistamines induced more drowsiness than a placebo, thus suggesting that such trials may mask potential subjective adverse effects. It was concluded that astemizole and terfenadine are essentially non-sedative antihistamines. Care should be exercised when evaluating potentially subjective adverse effects and trial design should be considered as a possible confounding effect.

**Humanistic - quality of life.** Tooley and colleagues[27] evaluated health-related quality of life (HRQOL) in patients with Chronic Obstructive Pulmonary Disease (COPD). HRQOL was assessed using the Chronic Respiratory Disease Questionnaire (CRDQ), a disease specific instrument, and the MOS SF-36, a general health survey. Each instrument was administered at baseline and at the end of the four week study period. In addition, the CRDQ was also administered at the end of week two. The average patient in this study was a 67 year old, white male with a 10 year history of COPD. Results of this study indicate that COPD patients experience significant impairment in HRQOL as measured by both the SF-36 and CRDQ.
CONCLUSIONS

Pharmacoepidemiology is a relatively young discipline with tremendous potential in enhancing the opportunities for gaining a better understanding of the resultant effects of pharmacotherapy. Caution is urged in viewing pharmacoepidemiologic studies, since the range of quality and validity of results need to be closely examined. Strom et al.[28] for example determined that the validity of data must be assessed before generalizations are made that may be erroneous. In their study, based solely on computerized data sources, there was a four-fold increase in risk of seizures in patients using transdermal scopolamine, but only in those patients who did not have preexisting seizures. After examining medical records, it was found that the data did not include rule out diagnosis for seizures, when this was taken into account, investigators were unable to find a single case of seizures after transdermal scopolamine in a patient who had not had seizures prior to receiving the drug. It was concluded that the data did not confirm the existence of an association between seizures and the use of transdermal scopolamine and demonstrates the importance of obtaining primary medical records when performing pharmacoepidemiologic studies with claims data.

Important variables in the future that will determine the ultimate utility of pharmacoepidemiologic studies include the need for enhanced communication between researchers, and a global view toward the analysis of data and study results. A multicontinent, transpopulation approach will enable a better dissemination of study findings, sharing of linked databases, and will lead to better patient care ultimately. Access to large data resources and the global linkage of these resources will be crucial defining aspects of the potential, future success of pharmacoepidemiology.
REFERENCES


[23] Neutel CI, Johansen HL. Perspectives on using record-linkage in pharmacoepidemiology. Post-Marketing-Surveillance 1993; 6(3-4); 159-173.


[27] Tooley JF, Cox FM, Parasuraman TV et al. Impact of chronic obstructive pulmonary disease on health-related quality of life. ASHP-Midyear-Clinical-Meeting; 1996, 31(Dec); P-349E.

FORMATION OF HETEROCYCLES IN THE MASS SPECTROMETER
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It was a misleading situation which stood at the beginning of our research on the formation of heterocycles in the mass spectrometer (Fig. 1): we had found traces of a naphthoindolizidine alkaloid in the asclepiadaceae plant cynanchum vincetoxicum, which shows a cis-configurated double bond in its $^1$H-NMR spectrum. Hydrogenation afforded a dihydro derivative which loses a four C-unit from the side chain, seemingly pointing towards benzylic cleavage of a C$_5$-side chain. This interpretation, however, was wrong. The combination of all the spectroscopic data showed that the complete side chain had been lost - against the fragmentation rules of mass spectrometry which “prohibit” a C-C cleavage at an aromatic C-atom, characterized by sp$^2$ hybridization.
We were misled again when we assumed that this fragmentation could be characteristic for naphthalene side chains, and then we speculated, that this abnormality might be due to the C-8 substituted tetrahydroisoquinoline skeleton, marked by bold lines in Fig. 1. Georg Stöber, a former coworker, was brave enough to synthesize C-8 substituted tetrahydroisoquinolines (THI) in spite of the difficulties well known to those who had ever been engaged in this field. He succeeded and mass spectrometry revealed that these THI do lose the complete side chain (Fig. 2). - To make a long story short, we proved that the THI was converted to a new heterocycle, an isoindoline by a mechanism shown in Fig. 3.
This is an example of an ortho-effect in mass spectrometry, characterized by irregular fragmentations of a specific increment due to the interference of ortho substituents. In our case a carbenium-immonium ion has been formed by β-cleavage as expected, followed by an electrophilic attack, analogous to a phenol-Mannich reaction, converting a sp²-hybridized C-atom into sp³-hybridized one which now is allowed to lose its substituent according to the rules of mass spectrometry.

Up to here, the results are published. The main topic of my lecture will be concerned with unpublished data of tetrahydroisoquinolines and aziridines, formed from aliphatic increments of oxime ethers.

Oxime ethers are interesting from a pharmaceutical point of view, because remedies of various types contain this increment (Fig. 4).

In the 70th Cooks and Varvoglis observed that oxim ethers with an O-alkyl group equal to n-propyl lose 30 amu. At the first glance this points towards a rearrangement to a nitron, which
subsequently loses NO (Fig. 5). High resolution MS, however, indicated that CH$_2$O was lost, followed by loss of a H-atom (Fig. 6). Cooks excluded the methine H to be involved in this processes. On the bases of these data these authors published a fragmentation mechanism shown in Fig.7: after transfer of a y-H- shorter alkyl chains do not show this rearrangement - formaldehyde is lost in a four membered transition state; the β-C is connected to the N, and in the subsequent step, H- is lost from the β-C, affording an ion at m/z 132.

Ph-CH=N-O-R$^+$ \[ \rightarrow \quad \text{Ph-CH-R}^+ \rightarrow \text{Ph-CH-}^+ \]

Fig 5

m/z 132 - structure 7

Ph-$\text{CH}=\text{N}=\text{H}$

m/z 105

Ph-$\text{CH}=\text{N}-\text{H}$

m/z 132
So far the results of Cooks and Varvoglis. - We thought, that their data are also in agreement with a postulation, binding the γ-C to the N (Fig. 8), and we shall realize, that this opportunity exists.

\[
\text{CH}=\text{N}-\text{O}-\text{CH}_2-\text{CH}_2-\text{CH}_3
\]

\[\alpha \beta \gamma\]

\[\text{H}^+ \text{migration}\]

\[
\text{CH}=\text{N}-\text{O}-\text{CH}_2-\text{CH}_2-\text{CH}_2
\]

\[\alpha \beta \gamma\]

\[-\text{CH}_2\text{O}\]

\[
\text{CH}=\text{N}-\text{O}-\text{CH}_2-\text{CH}_2-\text{CH}_2
\]

\[\alpha \beta \gamma\]

\[-\text{H}^+\]

\[m/z \ 132\]

Fig. 8

In order to clarify scope and limitation of this reaction we have checked the oxime ethers shown in Fig. 9. Compound 1 does lose 30 amu, but only with 1% intensity, and that is indeed NO. - 2 loses CH₂O, as already stated by Cooks, and so does the branched cpd. 3. Because the α-C carries a methyl group, C₂H₄O is lost instead of CH₂O in cpd. 4. The n-butyl- and n-pentyl ethers (cpds. 5 and 6) behave as expected, however, the intensity of (M - 30)⁺ ions is higher because the remaining C-radical is stabilized by an alkyl group. This tendency is still increased in cpd. 7 (tert. radical), whilst cpd. 8 (no γ-H) does not lose 30 amu. All these findings point toward the γ-H- migration to be the rate determining step.
As to find out the origin of the migrating H, as well as of that H which is lost after the loss of CH$_2$O, so leading to the ion at m/z 132, we have specifically deuteriated the positions shown in Fig. 10. There is a dominant loss of D from the ring, pointing towards a ring closure by radical attack of the γ-C-radical which has been generated by γ-H transfer to the N atom. This will be scrutinized below. Cpd. 3a loses 32 amu, id est CD$_2$O, in accordance with Cooks, but - contrary to Cooks - 3b does not lose the D- located in the β-position. In the sixfold deuteriated cpd. 3c all the D remains in the fragments (M - 31)$^+$. 
I have mentioned that the transfer of γ-H might be the rate determining step. This was proven by a type of experiment, inaugurated by Schwarz, making use of the inverse isotope effect (Fig. 11). Way a indicates migration of H-, leading to a C-CH₂ radical, which loses -CD₃ for stabilization. Following way b, D- is transferred to N, leaving a C-CD₂ radical behind. After loss of formaldehyde -CH₃ is lost. Consequently, migration of D- results in loss of deuterated methyl radical and vice versa. This explains the attribute „reverse“ isotope effect. Because the mass of D is twofold that of H, migration of D- is impeded as compared to that of H. This difference, however, will only become evident, if this migration is the rate determining step. This holds true for our problem, the ratio \( k_H/k_D = (M - 30 - ³\text{CD}_3)⁺ / (M - 30 - ³\text{CH}_3)⁺ \) is 2.1.

Concerning the origin of D-, eliminated after the loss of formaldehyde, Fig. 12 compiles our findings: 96 % of D- stem from the aromatic ring, and D- is split off from all aromatic positions. This has been proven by experiments with specifically labeled benzaldehydes as starting materials.
Fig. 12 explains the formation of the 3,4-dihydroisoquinoline cation representing the structure of m/z 132 [(M⁺ - CH₂O - H)⁺]. The loss of D from the m- and p-positions is explained by a sequence of 1,2-D shifts. Analogously, ⁶⁺Cl is ejected from all aromatic positions.
The 3,4-dihydroisoquinolinium structure was proven by comparison of the CAD spectra of the ion under consideration with those obtained from C-1 substituted tetrahydroisoquinolines (Fig. 14). Benzylic cleavage, especially favoured in this case because it is also a β-cleavage, definitely affords the 3,4-dihydroisoquinolinium ion, and the CAD (Collisionally Activated Dissociation) spectra (Fig. 15, e.g.) reveal that the spectra are very similar but not identical. The small but obvious deviations may indicate that the ion m/z 132 includes an additional isomeric compound (Fig. 16). Because the methine-H is lost to a low extent, the azetidine radical cation to be discussed below is converted to the azetine cation, an isomer of the 3,4-dihydroisoquinolinium ion. Moreover, the inherent energy content of the 3,4-dihydroisoquinoline cations is slightly different, depending on the tetrahydroisoquinoline precursors, because even the dihydroisoquinoline cations obtained from different tetrahydroisoquinoline precursors are not similar in every detail (data not shown).
Fig. 14

Fig. 15
The postulation of a 2-phenylazetidine intermediate became necessary in order to explain the formation of the ions at m/z 105 and m/z 104, generated by 2+2-cycloreversions (Fig. 17). In order to prove this azetidine hypothesis we synthesized 2-phenylazetidine and 4-methyl-2-phenylazetidine (the latter one was used for comparison with the homologous n-butyl ether of benzaldoxime). After addition of \( \text{IN}_3 \) to phenyl-2-propene, HI was eliminated, and dichlorocarbene was added to the double bond (Fig. 18) following Hassner’s procedure.
Thermal formation of the nitrene and its insertion gave rise to the dichloro-phenyl-methylazetine which was reduced and dehalogenated by LiAlH₄. Comparison of the CAD spectra of the (M - 30)+ ion of the n-butyl ether mentioned above and that of the azetidine showed, that all the signals of the azetidine are present, but some of the intensities are different. In the case of the propyl ether (Fig. 19), however, the spectra of the (M - 30)+ ion (top) and that of the 2-phenylazetidine (bottom) are virtually identical. It turned out from experiments with the fluoreninoxime n-butyl ether shown in Fig. 20 that an ion dipole complex a is formed which is in equilibrium with two azetidine isomers b and c. In the spiro-heterocycle b the γ-C carrying the terminating methyl group of the former butyl increment is connected to the N-atom (cf. Fig. 8), whilst in the isomer c the β-C is bound to the N, leading to a less crowded molecule, because the methyl group is apart from the bulky fluorene increment. If we assume, that the energy differences of these isomeric spiro heterocycles reflect the activation energy for their formations we can easily understand the differences in the CAD spectra. This is not mere speculation because the propyl ether, specifically labelled by D in the β-position revealed fragments a and b (Fig. 21) generated by the 2+2-cycloreversion of the two spiro-azetidines, carrying D or H in the alkene fragments a or b, respectively.
Fig. 18

Fig. 19
The approaching of either the β-C or the γ-C to the N-atom is strongly influenced by the bulkiness of the γ-substituent. Our data indicate that the γ-C is nearly exclusively bound to the N-atom in the case of the bulky phenyl ring, whilst the β- or γ-position is bound to the N atom with the γ-substituent being a methyl group.

The last aspect to be discussed in the context of ring formation from aliphatic molecular increments describes the necessity to explain why the terminating methyl group in the n-butyl benzaldoxime ether is lost after γ-H migration: this should be impossible for the n-butyl ether A, whilst it can easily be rationalized in the isomeric β-methylpropyl ether B (Fig.22). Moreover, we have to explain why the n-butyl ether A and the iso-butyl ether B give virtually identical mass spectra. As it can be rationalized from the corresponding isomeric methyl-phenylazetidines, loss of CH₃ should be dominant in B', but of minor importance in A'. In fact, however, both mass spectra are nearly identical, and this again points towards an equilibrium of A' and B' in the gas phase via an ion dipole complex.

The formation of heterocycles from aliphatic molecule increments had been observed already by other groups (Grützmacher et al., e.g.); for us the elucidation of these reaction mechanism was and still is an exciting task and I do hope that I could transfer some of this excitement to you.
Fig. 22
TRADITIONAL FOLK MEDICATIONS & NATURAL RESOURCES FOR ADULT-DISEASE PREVENTION

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ABSTRACT

It has become apparent that environmental factors play an important role in causing and in preventing adult diseases. Prevention of disease in adults is now the most urgent public health problem such as human heart disease, cancer and diabetes, which is associated with a variety of vascular complications.

Keywords: adult disease, hematological activity, two-stage mouse lung carcinogenesis, two-stage skin carcinogenesis, Turkish medicinal plant, spice, Umbelliferous plant, Allium spp.

INTRODUCTION

Human cancer development and some adult disease depend on daily life style as living environment, smoking, foods, etc. We have been studied surveying ways to evaluate the prevention on the complications of diabetes, hematological activity, cytotoxic and antitumor-promoting activity by many kinds of natural materials in vitro and in vivo systems. Many compounds markedly inhibited the incorporation of $^3$H-choline and $^{32}$P incorporation into phospholipids of cultured cells. Moreover, some coumarins, chalcones, triterpenoids, saponins were proved the anti-tumor-promoting activity in mouse skin carcinogenesis induced for two-stage mouse lung, skin and colon carcinogenesis.

In this symposium, I want to discuss the chemical studies on Turkish medicinal plants, moreover our recent research on three kinds of bioassays by next materials, Umbelliferous plants as Chinese crude drugs, Qian-Hu: Peucedanum praeruptorum and P. decursivum, Angelicae Radix: Angelica acutiloba var. sugiyamae and edible plant such as Asshita-Ba in Japanese,
Angelica keiskei and Traditional folk medicine, Ferula elaeactytis, Ferulago trachycarpa and Frangos platychaena in Turkey and Allium spp., Garlic, A. sativum, and Xiebai, Allium chinense as spices.

EXPERIMENTS

Platelet Aggregation --- Fresh blood, obtained from a healthy human and Japanese white male rabbit containing 3.8% sodium citrate were prepared under the ordinary method [1] to obtain a supernatant of platelet-rich plasma (PRP) and Platelet-poor plasma (PPP). Each sample was dissolved in saline or methanol and 5 µl of each was added to 195 µl PRP. The final concentrations of adenosine 5'-diphosphate (ADP) as inducers was at 2 µM. Platelet aggregation was determined by a turbidimetric method using a platelet aggregometer (Mebanix).

Measurement of Blood Coagulation Time --- This test was assayed using our method obtained from a healthy rabbit [1]. Saline or DMSO was used as a control.

Assay of Fibrinolysis --- This experiment was carried out as described our papers [1]. The same method was used for the calculation of the IC50 values of active samples.

Assay of Bovine-LAR Activity --- Bovine-LAR was prepared by our method [2]. Each sample was tested for aldose reductase activity at concentrations of 30, 10 and/or 3 µg/ml in DMSO and the presence of 0.1% DMSO in the final assay mixture.

32P Incorporation into phospholipid of cultured cells --- Incorporation of 32Pi into phospholipids of HeLa cells, which were cultured in Eagle's minimum essential medium supplemented with 10% calf serum under a humidified atmosphere of 5% CO2 in air [3].

Assay for 3H-Choline Incorporation into Phospholipids of Cultured Cells --- Incorporation of 3H-choline into phospholipids of CH10T1/2 cells was assayed as our method [3]. Each extract was dissolved in DMSO to give a corresponding test solution, which was applied to CH10T1/2 cells. Mouse fibroblast CH10T1/2 cells were incubated with 50 mg/ml of each test solution 3H-choline (370 kBq/culture) was added with or without TPA (50 nM), and the radioactivity incorporated into phospholipids of cells were measured.
Assay for Epstein-Barr virus (EBV) induction — The detection of EBV-EA positive cells was carried according to the method of Ito et al. [4]. Raji cells, an EBV genome-carrying human lymphoblastoid cells, were incubated for 48 hr in 1 ml of medium containing β-butyric acid (88 mg), TPA (20 ng) and various concentrations of samples. The induction rate of early antigen (EA) of EBV was compared to that of a control experiment, which was incubated only with β-butyric acid and TPA.

TPA-Induced Mouse Ear Edema — Male ddY and female ICR mice at 6 weeks of age were assigned to groups, housed together, and acclimatized under standard conditions with free access to food and water for a week. Each 20 ml of test solution was applied topically to both inner and outer surfaces of the right ear only using a micropipette. After a 30-min, treatment with the test solution, each ear was then treated with TPA dissolved in acetone at 100 mg/ml, 2 mg per ear, in the same manner. Ear thickness was measured 5 h after TPA treatment.

Assay for Inhibition of Ornithine Decarboxylase (ODC) — Induction of ODC was carried out by a single application of 10 mg (16.5 n mol) TRA on the back of 7-weeks-old male ICR mice. Sample (molar ratio to TPA was 10:1) was applied with or without TPA. Blank group was treated with 200 ml of acetone, which was used as the solvent for TPA and sample. After 4hr, epidermis was collected in the buffer. The samples were sonificated and ultracentrifuged at 32,000 rpm for 20 min. Cell extracts were held on ice until the ODC assay was performed.

Two-Stage Carcinogenesis Experiments:

(1) Two stage mouse skin carcinogenesis — The back of six-week old ICR female mouse was shaved, which were carcinogenetically initiated with DMBA 150 mg (585 nmol) in acetone (0.1 ml) topically. One week after initiation, TPA (2 mg, 3.25 nmol) in acetone was applied on the same part of the back of mice twice a week. Each sample was administrated by topically and orally. Sample topical treated group was applied at the dose 1 mg per mouse, 30 min. early for each TPA-treatment. In oral administration group, sample was dissolved in drinking water (1.25 mg/100 ml) , and given ad libitum for promotion period. TPA and test sample were continuously applied for 18 weeks.
(2) Two stage mouse lung carcinogenesis experiments --- 4 NQO as an initiator was dissolved in a mixture of olive oil and cholesterol (20:1), of which 0.3 mg (1.57 m mol) /mouse (6 weeks old male ddY mice) was given by single subcutaneous (s. c.) application at the starting time. A 5% glycerol in water as a promoter was given as drinking water ad libitum from the beginning of experimental week 5 continuously for 25 weeks. Mice as control group were given 5% glycerol without HCO or with HCO 60. Mice was killed at week 30 by cervical dislocation of 10% formaldehyde. After separation of each pulmonary lobe, the number of induced tumors was counted under a microscope.

RESULTS AND DISCUSSION

I. Chemical Studies on Turkish Medicinal Plants

We have examined the course of going studies to establish the useful on Egyptian [4] and Turkish traditional folk medicines. I will discuss the chemical constituents isolated from three kinds of Umbelliferous plants collected in Turkey with Prof. M. Coskun as co-workers as shown in Fig. 1. The purification of other minor components and the biochemical activities are now in progress.

II. Anti-Blood Coagulation of Compounds from Chinese Plants used for Thrombosis-like Diseases

In the course of searching for potent anti-thrombotic and/or anti-tumor agents, a number of naturally occurring compounds were isolated from Chinese medicinal plants and insects [14]. Here is the results of screening tests on platelet aggregation, blood coagulation and fibrinolysis by Allium species. Sulfur containing compounds (15,16), acid amides, N-p-coumaryltyramine (17) and N-trans-feruloyltyramine (18), eight kinds of saponins and sapogenin, laxoside (19), xiebai-saponin I (20), chinemosides II (21), III (22), macrostemonoside A (23), proto-iso-eruboside B (24), iso-eruboside B (25), laxogenin (26) and two chalcones, isoliquiritigenin (27) and liquiritigenin (28), and adenosine (29) were isolated from "Xiebai" (Japanese name Gaibaku), the tuber of Allium chinense, A. macrostemon, and "Garlic", A. sativum, respectively. Acid amides (17,18) and 29 were showed a significant inhibitory activity against human platelet aggregation by 2 mM ADP [1e]. Compounds 23, 25 and 29 prolonged the coagulation time significantly and 21, 22, 24, 25 promoted fibrinolysis activity as shown in Table 1(11).
Fig. 1 Structures of Compounds Isolated from Turkish Umbelliferae Plants

*Prangos platychilaena* (1-8), *Ferula amanica* (1-3, 9-11) and *Ferulago* spp (2, 10, 12-14).
Fig. 2. Structures of Compounds Isolated from Xiebai (Allium chinense and A. macrostemon)
### TABLE 1. Effects of test Compounds on Blood Coagulability

<table>
<thead>
<tr>
<th>Sample</th>
<th>Platelet aggregation effect (%)</th>
<th>Prolongation effect of coagulation (%)</th>
<th>Fibrinolysis promotive effect (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ±S.D. IC&lt;sub&gt;50&lt;/sub&gt;(µg/ml)</td>
<td>Mean ±S.D. IC&lt;sub&gt;50&lt;/sub&gt;(µg/ml)</td>
<td>Mean ±S.D. ED&lt;sub&gt;50&lt;/sub&gt;(mg/ml)</td>
</tr>
<tr>
<td>laxoside</td>
<td>-27.6±2.3*</td>
<td>213.0±8.9*</td>
<td>-15.0±4.6</td>
</tr>
<tr>
<td>chinenoside II</td>
<td>7.0±0.0*</td>
<td>-13.3±12.2</td>
<td>80.0±19.8</td>
</tr>
<tr>
<td>chinenoside III</td>
<td>8.0±3.2*</td>
<td>-6.0±6.2</td>
<td>90.0±20.9</td>
</tr>
<tr>
<td>macrostemonoside A</td>
<td>0.5±0.5</td>
<td>999*</td>
<td>8.5±9.3</td>
</tr>
<tr>
<td>proto-iso-eruboside B</td>
<td>-2.5±4.4</td>
<td>5.0±5.5</td>
<td>78.0±23.6</td>
</tr>
<tr>
<td>iso-eruboside B</td>
<td>-27.6±2.3*</td>
<td>999*</td>
<td>55.8±15.7</td>
</tr>
<tr>
<td>adenosine</td>
<td>-71.6±5.3*</td>
<td>-10.3±9.8</td>
<td>-34.5±8.4*</td>
</tr>
<tr>
<td>allin</td>
<td>3.3±2.8</td>
<td>-4.0±13.9</td>
<td>-43.3±3.5*</td>
</tr>
<tr>
<td>(+) S-allyl cysteine</td>
<td>2.03±4.0</td>
<td>-14.7±8.7</td>
<td>5.0±14.0</td>
</tr>
</tbody>
</table>

*Significantly different from the control by Student's t-test (P<0.01): a : final concentration=2 µM, b : IC<sub>50</sub> values of fibrinolysis-promoting effect are the concentrations in sample solution, not the concentrations in reaction mixtures (final concentrations), c : 999 means that the sample does not coagulate within 20 min.
III. Search to Prevent the Complications of Diabetes

1) Inhibitory Effect by Coumarin and Flavonoid Derivatives on Bovine Lens Aldose

Increased aldose reductase (AR) activity and platelet aggregation are considered as causes of diabetic complications. Sorbitol is produced from glucose by AR through the polyol pathway and in diabetes, the substantial accumulation of sorbitol in cells causes damage. This is associated with the development of some chronic diabetic complications, such as cataracts, neuropathy and retinopathy. Also diabetes mellitus is associated with a variety of vascular complications like myocardial infarction and peripheral vessel disease. We described their inhibitory effects on both bovine-LAR and rabbit platelet aggregation by coumarins, flavonoids and polyphenol compounds [2].

IV Anti-tumor-promoting Activity in vitro and in vivo systems

We are approaching the chemoprevention applied to the promoting stage of carcinogenesis by traditional folk medicines. Now, we carried out the screening tests in vitro and in vivo systems to obtain many useful materials from Chinese crude drugs, Umbelliferous plants and Allium spp.

in vitro system
1. $^{32}$P incorporation into phospholipid in HeLa cells
2. $^3$H-Choline Incorporation into Phospholipids in C3H10T1/2 cell
3. Epstein-Barr virus (EBV) activation in Raji cells
4. Apoptosis

in vivo system
1. TPA-Induced Mouse Ear Edema
2. Ornithine Decarboxylase (ODC) activity
3. Azoxymethane-induced Aberrant Crypt Foci (ACF)
4. Two-Stage Mouse Skin Carcinogenesis: initiator DMBA, promoter TPA
5. Two-Stage Mouse lung Carcinogenesis: initiator 4-NQO, promoter glycerol

Effect of Coumarins isolated from Chinese crude drugs, Tang-Bai-Zhi, Angelicae Radix and Xian-Hu

1. By monitoring for activity on TPA-enhanced radioactive inorganic phosphate ($^{32}$P) incorporation into phospholipids of HeLa cells, six kinds of linear-type furanocoumarins having a dimethylallyl or a related group linked through an ether bond at the C5 and/or C8 positions,
isolated from Chinese Crude Drug "Tang-Bai-Zhi", the root of Angelica dahurica (Table II)[3b, d])

TABLE II. Inhibitory Effect of Coumarines obtained from "Tang-Bai-Zai" and "Ashita-Ba" on TPA-Enhanced 32Pi-Incorporation into phospholipids of HeLa Cells. (TPA: 50nM)

<table>
<thead>
<tr>
<th>Coumarins (50 μg/ml)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>imperatoin</td>
<td>86.5</td>
</tr>
<tr>
<td>isoimperatoin</td>
<td>94.1</td>
</tr>
<tr>
<td>oxypeucedanin</td>
<td>31.9</td>
</tr>
<tr>
<td>Pabulenol</td>
<td>36.7</td>
</tr>
<tr>
<td>byakangelcin</td>
<td>0</td>
</tr>
<tr>
<td>neobyakangelcin</td>
<td>2.9</td>
</tr>
<tr>
<td>psoralen</td>
<td>12.9</td>
</tr>
<tr>
<td>bergapten</td>
<td>15.7</td>
</tr>
<tr>
<td>xanthotoxin</td>
<td>23.8</td>
</tr>
</tbody>
</table>

2. The ID50 values of two linear pyranocoumarins, acutilobin and decursin, from "Angelicae Radix", the root of Angelica acutiloba var. sugiyamae, were about 3 μg/ml and 6 μg/m, respectively, and the inhibiting effect of acutilobin was stronger than that of known antitumor-promoterers, such as quercetin [3c]).
3. We have been studied the constituents of "Qian-Hu", the root of *Peucedanum praeruptorum* and *P. decursivum* to isolate 30 coumarins and 5 saponins. Some structure-activity relationships among Pd-Ia, Pd-II and the related coumarins can be deduced on TPA-enhanced $^{32}$Pi incorporation into phospholipids of HeLa cells (Table III). Pd-II was studied for the effect on TPA-induced early antigen (EA) of EBV in Raji cells. This assay system allows for a rapid detection of the activity of the tumor promoter such as TPA. In addition, Pd-Ia and Pd-II were tested for their effects on two-stage mouse skin (see Fig. 3) and lung carcinogenesis experiments [3g]).

**TABLE III. Effect of Pd-II-Related Compounds on TPA-Enhanced $^{32}$Pi Incorporation into Phospholipids of HeLa Cells. (TPA, 50nM)**

<table>
<thead>
<tr>
<th>Source</th>
<th>Compounds</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bai-Hua Qian-Hu (Q-I type)</td>
<td>Pd-Ia</td>
<td>84.7</td>
</tr>
<tr>
<td></td>
<td>Pd-II</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Pd-III</td>
<td>100</td>
</tr>
<tr>
<td>Bai-Hua Qian-Hu (Q-II type)</td>
<td>peucedanocoumarin I</td>
<td>44.5</td>
</tr>
<tr>
<td></td>
<td>peucedanocoumarin II</td>
<td>76.1</td>
</tr>
<tr>
<td></td>
<td>peucedanocoumarin III</td>
<td>57.9</td>
</tr>
<tr>
<td></td>
<td>pteryxin</td>
<td>86.7</td>
</tr>
<tr>
<td>Zi-Hua Qian-Hu (Q-III type)</td>
<td>decursidin</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Pd-C-IV</td>
<td>63.7</td>
</tr>
<tr>
<td></td>
<td>nodakenetin</td>
<td>25.1</td>
</tr>
<tr>
<td>Coumarin derivatives prepared by chemical modification</td>
<td>Pd-III-4'-OH</td>
<td>95.8</td>
</tr>
<tr>
<td></td>
<td>(+)-cis-khellactone</td>
<td>47.0</td>
</tr>
<tr>
<td></td>
<td>(+)-trans-khellactone</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td>khellactone-4'-OCH$_2$NH$_2$</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>Pd-Ia-4'-OCH$_2$NH$_2$</td>
<td>46.3</td>
</tr>
</tbody>
</table>

HeLa cells were incubated with one of the test compounds (50 µg/ml) for 1 h, and then $^{32}$Pi was added with or without TPA (50 nM). Incubation was continued for 4 h, and then the radioactivity incorporated into phospholipids was assayed.
Fig. 3. Effect of Pd-II on the Promotion of Skin Tumor Formation by TPA in DMBA-Initiated Mice in Vivo

From 1 week after initiation by a single application of 100μg of DMBA, 0.5μg of TPA was applied twice a week. Pd-II (10μmol) or glycyrrhetinic acid (10μmol), was applied 40min before TPA application. Percentage shows the tumor-bearing mice.

Control group: treated with DMBA+TPA
Sample group: treated with DMBA+TPA+Pd-II
 treated with DMBA+TPA+glycyrrhetinic acid.
**Anti-tumor-promotion by principles obtained from Angelica keiskei**

From the active fraction of "Ashita-Ba", *Angelica keiskei*, edible in Japan, five angular pyranocoumarins and three chalcones, 4-hydroxyderricin, xanthoangelol and ashitaba-chalcone were isolated. Among these compounds, 4-hydroxyderricin and xanthoangelol were proved to have anti-tumor-promoting activity in mouse skin carcinogenesis [3e].

**Antitumor-promoting activity of spices**

Spices have been widely used throughout the daily foodstuffs, pigments, perfumes and condiments, in some cases as traditional folk medicines. We prepared four kinds of extracts from 37 spices, which extracts were examined for antitumor-promoting activity on TPA-enhanced $^3$H-choline incorporation into phospholipids of C3H10T1/2 cells *in vitro* and on TPA-induced mouse ear edema *in vivo*.

Among the tested spices, Basil, Ginger, Marjoram, Rosemary, White pepper and Xiebai significantly inhibited TPA-enhanced $^3$H-choline incorporation into phospholipids of C3H10T1/2 cells. Allspice, Basil, Bay (Laurel), Cardamom seed, Cinnamon, Cumin, Dill seed, Dry ginger, Ginger, Japanese parsley, Horseradish, Marjoram, Oregano, Parsley, Pink pepper, Red pepper, Rosemary, Sage, Tarragon, Thyme, Turmeric and White pepper were highly potent inhibitors of TPA-induced mouse ear edema. Each compound, ursolic acid from Sage, luteolin from Celery seed, laxogenin from Xiebai and piperine from White pepper, along with capsaicin, a main constituent of Red pepper, exhibited TPA-induced mouse ear edema (Table IV) [3i].

**TABLE IV. Inhibitory Effect of Compounds on TPA-Induced Mouse Ear Edema**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Dose (mg)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ursolic acid</td>
<td>1.0mg</td>
<td>54.4</td>
</tr>
<tr>
<td>Luteolin</td>
<td>1.0mg</td>
<td>80.6</td>
</tr>
<tr>
<td>Laxogenin</td>
<td>1.0mg</td>
<td>24.5</td>
</tr>
<tr>
<td>Piperine</td>
<td>3.0mg</td>
<td>63.2</td>
</tr>
<tr>
<td>Capsaicin</td>
<td>1.0mg</td>
<td>25.8</td>
</tr>
<tr>
<td>Indomethacine</td>
<td>1.0mg</td>
<td>84.8</td>
</tr>
</tbody>
</table>

Each compound was applied topically 30 min before TPA (2mg/ear) treatment. Ear thickness was determined 5h after TPA treatment.
The acetone soluble part and the thin crushed powder of White pepper, the fruits of *Piper nigrum* L. (Piperaceae), were proved to have antitumor-promoting activity in two-stage mouse skin carcinogenesis induced by DMBA as initiator plus TPA as promoter, in addition, in the two-stage mouse lung carcinogenesis induced by 4 NQO as initiator plus glycerol as promoter. The topical treatment of the thin powder prepared from White pepper was affected the effect of decrease and in delaying emergence of skin tumor formation, although that of piperine was no so high potency [31].

**Antitumor-promoting activity by "Xiebai"**

Two saponins, laxoside (19) and xiebai-saponin I (20) with their aglycone, laxogenein (26), and chalcones (27,28) were evaluated an inhibitory effect on TPA-stimulated incorporation into phospholipids of cultured cells in vitro experiment. Among tested compounds, isoliquiritigenin (27) exhibited significant inhibitory effect. In addition, two-stage mouse lung of 26 and two stage lung and skin carcinogenesis of 27 were proved to have antitumor-promoting activity.

During the course of ongoing studies to establish the effective against chemical carcinogenesis in different organ and by different experimental method, we have proposed an in vivo short-term assay using azoxymethane (AOM)-induced aberrant crypt foci (ACF), and cell proliferation intermediate biomarkers, such as ornithine decarboxylase (ODC) activity in epithelium of the colon in rats and mice.
REFERENCES AND NOTES


METABOLISM OF ANANDAMIDE, AN ENDOGENOUS LIGAND FOR CANNABINOID RECEPTORS

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ABSTRACT

Anandamide (arachidonylethanolamide) is an endogenous ligand for cannabinoid receptors, and its biological activities are lost by its enzymatic hydrolysis to arachidonic acid and ethanolamine. We partially purified "anandamide amidohydrolase" from the microsomes of porcine brain to a specific activity of 0.37 mmol/min/mg protein by hydrophobic chromatography. The enzyme preparation catalyzed not only the hydrolysis of anandamide but also its synthesis by the reverse reaction. Several lines of enzymological evidence suggested that the two reactions were catalyzed by a single enzyme protein. This finding was confirmed with a recombinant enzyme transiently expressed in COS-7 cells by the use of the recently cloned cDNA for the rat enzyme hydrolyzing oleamide. We also examined the tissue distribution of the enzyme in rat. Liver showed by far the highest activities of anandamide synthase and hydrolase. We noted that the hydrolase activity was much lower than the synthase activity in small intestine. However, the low activity was attributable to inhibition by endogenous lipids, and after removal of lipids by acetone extraction the hydrolase activity in small intestine was as high as the synthase activity. Furthermore, by Northern blot analysis an intense band of the anandamide amidohydrolase mRNA was detected in small intestine and stomach as well as liver. These results demonstrated the presence of a considerable amount of the enzyme in small intestine.

Key words: Marijuana, Cannabinoid, Anandamide, Arachidonic acid, Amidohydrolase
INTRODUCTION

Cannabinoids are psychoactive components contained in marijuana, and D9-tetrahydrocannabinol, a representative of cannabinoids, was isolated in 1964 (Figure-1) [1]. Later, a cannabinoid-specific receptor was found in the membrane fraction of rat brain [2], and its cDNA was cloned from rat brain [3]. cDNA for a peripheral type of cannabinoid receptor was also cloned from human promyelocytic leukemia cells HL60 [4]. The central type and peripheral type of cannabinoid receptors are now referred to as CB1 and CB2, respectively.

Figure-1. Chemical structures of D9-tetrahydrocannabinol (A) and anandamide (B).

In 1992 an endogenous ligand for cannabinoid receptors was isolated from porcine brain [5]. The compound was identified as arachidonylethanolamide, and referred to as anandamide (Figure-1). Anandamide binds to cannabinoid receptors, reduces the intracellular cAMP concentration by activating pertussis toxin-sensitive G protein, and inhibits N-type calcium channels through pertussis toxin-sensitive G protein [for review, 6 and 7]. A variety of cannabimimetic pharmacological and behavioral activities of anandamide were also reported [6,7].
Biological activities of anandamide are lost by its enzymatic hydrolysis to arachidonic acid and ethanolamine [8] (Figure-2). The “anandamide amidohydrolase” activity was found in several mammalian cell lines and tissues such as brain and liver [8-15]. The enzyme activity was found mostly in particulate fractions like microsomes. On the other hand, anandamide could be formed by the condensation of arachidonic acid and ethanolamine, and the “anandamide synthase” activity was found in brain and other tissues [8,10,13,15-18]. The enzymological characterization of the anandamide amidohydrolase and its tissue distribution will be discussed in this chapter.

\[
\begin{align*}
\text{Anandamide} & \xrightarrow{\text{H}_2\text{O}} \text{H}_2\text{N} \xrightarrow{\text{OH}} \text{Ethanolamine} \\
& \xrightarrow{\text{H}_2\text{N} \xrightarrow{\text{OH}} \text{Arachidonic Acid}}
\end{align*}
\]

Figure-2. The reaction catalyzed by anandamide amidohydrolase.

1. Partial purification and characterization of anandamide amidohydrolase from porcine brain

Porcine brain was obtained at a local slaughterhouse, and homogenized with a Potter-Elvehjem homogenizer in 9 times the volume of 20 mM Tris-HCl buffer (pH 8.0) containing 0.32 M sucrose. A microsomal fraction (105,000 x g pellet) was prepared by ultracentrifugation, and treated with 1% Triton X-100 at 4 oC for 12 h. The sample was then subjected to ultracentrifugation, and the resultant supernatant was used as solubilized proteins. For the anandamide hydrolase assay, the solubilized proteins were incubated with 100 μM
[14C]anandamide (10,000 cpm/5 µl of ethanol) in 200 µl of 50 mM Tris-HCl buffer (pH 9.0) for 20 min at 37 °C, and radioactive compounds were extracted with diethyl ether, followed by thin-layer chromatography. When the distribution of radioactivity on the plate was scanned by a Fujix bioimaging scanner BAS2000, the band corresponding to [14C]arachidonic acid was detected. The heat-treated proteins did not produce arachidonic acid. For the anandamide synthase assay, the proteins were allowed to react with 250 µM [14C]arachidonic acid (50,000 cpm/5 µl of ethanol) in 200 µl of 250 mM ethanolamine-HCl buffer (pH 9.0) for 20 min at 37 °C. As examined by thin-layer chromatography, anandamide was formed in the presence of ethanolamine, but not in its absence. These results showed that the solubilized proteins had the anandamide hydrolase and synthase activities.

We tried to purify the hydrolase, but several conventional column chromatographies did not bring about its efficient purification. A satisfactory result was obtained by hydrophobic chromatography with a Tosoh Phenyl-5PW column (Tokyo, Japan). As shown in Figure-3, the solubilized proteins were applied directly onto the column, and proteins were eluted by reducing the concentration of ammonium sulfate. A bulk of proteins was eluted in Peak I while

Figure-3. Partial purification of anandamide amidohydrolase of porcine brain by Phenyl-5PW hydrophobic chromatography. Closed circles, the anandamide hydrolase activity; open circles, the anandamide synthase activity.
the anandamide hydrolase activity was detected in Peaks I and II. By this procedure the enzyme eluted in Peak II was purified by about 20 fold, and the specific hydrolase activity of the partially purified enzyme was 0.37 μmol/min/mg protein at 37 oC. We noted that the anandamide synthase activity was co-eluted with the hydrolase activity (Figure-3). The specific synthase activity in Peak II was 0.16 μmol/min/mg protein. When the Peak II fraction was applied onto a Tosoh DEAE-5PW ion-exchange column, the hydrolase and synthase activities were co-eluted again, suggesting that the two reactions were derived from a single enzyme protein. We used the Peak II fraction as a partially purified enzyme in the following experiments.

The hydrolase and synthase reactions proceeded linearly up to 20 min, and the activities were dependent on the amounts of enzyme protein. Optimal pH was 7.5 - 9.0 for both the reactions. The apparent Km value for anandamide was approximately 60 μM. As compared at 300 μM, the enzyme hydrolyzed ethanolamides of linoleic acid, oleic acid and palmitic acid at 44 %, 27 % and 19 % of the rate of the anandamide hydrolysis. This wide substrate specificity suggests that anandamide amidohydrolase is identical to N-acylethanolamine amidohydrolase, which was earlier shown to be reactive with various fatty acid ethanolamides [19]. When the synthase activity was examined in the presence of ethanolamine, the enzyme converted arachidonic, linoleic, oleic, and palmitic acids to their ethanolamides, and the reaction rates were not very different between these fatty acids. The apparent Km value for arachidonic acid was about 100 μM, and that for ethanolamine was as high as about 50 mM.

We also tested several compounds for inhibitory effects on the hydrolase and synthase activities (Figure-4). Arachidonoyl trifluoromethyl ketone (ATK) was reported as a cytosolic phospholipase A2 inhibitor and also shown to inhibit anandamide amidohydrolase [20]. This compound inhibited the hydrolase and synthase activities in parallel with IC50 values around 1μM. Serine protease inhibitors like phenylmethylsulfonyl fluoride (PMSF) and diisopropyl fluorophosphate (DFP) and a sulfhydryl-reactive agent, p-chloromercuribenzoic acid (PCMB)
also inhibited both the activities. All these results suggest that the anandamide hydrolase and synthase activities are derived from a single enzyme protein [10].

Figure-4. Inhibition of the anandamide hydrolase and synthase activities by various compounds.

2. Expression of rat anandamide amidohydrolase cDNA in mammalian cells

Primary amide of oleic acid (oleamide) was suggested to be an endogenous sleep inducer, and shown to be hydrolyzed enzymatically [21]. In 1996 Cravatt et al. cloned cDNA for oleamide amidohydrolase from a rat liver cDNA library [22]. They found that the recombinant enzyme hydrolyzed not only oleamide but also anandamide, and referred to the enzyme as fatty-acid amide hydrolase [22]. It was also reported that anandamide amidohydrolase of N18 mouse neuroblastoma cells was reactive with oleamide [23]. Thus, fatty-acid amide hydrolase appears to be identical to anandamide amidohydrolase.
On the basis of the reported cDNA sequence, we obtained a cDNA for fatty-acid amide hydrolase by reverse-transcriptase polymerase chain reaction using rat liver mRNA as a template. The cDNA was inserted to an eukaryotic expression vector pcDNA3.1(+) (Invitrogen), and COS-7 cells were transfected with the vector. After 3 days the cells were harvested and sonicated, and the particulate fraction was prepared by ultracentrifugation. When the particulate fraction was allowed to react with [14C]anandamide, [14C]arachidonic acid was produced depending on the concentrations of anandamide (Figure-5A). The specific enzyme activity was 132 nmol/min/mg protein at 37 °C, and the Km value for anandamide was about 20 μM. The control cells, transfected with the insert-free vector, did not show the hydrolase activity. When the enzyme was incubated with [14C]arachidonic acid in the presence of ethanolamine, [14C]anandamide was produced depending on the concentrations of the substrates (Figure-5B and 5C).

Figure-5. The anandamide hydrolase and synthase activities of the recombinant enzyme expressed in COS-7 cells. The recombinant enzyme was allowed to react with different concentrations of [14C]anandamide (A), with different concentrations of [14C]arachidonic acid in the presence of 250 mM ethanolamine (B) or with 200 μM [14C]arachidonic acid in the presence of different concentrations of ethanolamine (C).
The specific synthase activity was 177 nmol/min/mg protein. The Km values for arachidonic acid and ethanolamine were 190 μM and 36 mM, respectively. The control cells did not synthesize anandamide. These results confirmed the reversibility of the reaction catalyzed by anandamide amidohydrolase. However, due to the extremely high Km value for ethanolamine, the enzyme appears to act as hydrolase rather than synthase under the physiological conditions. Another biosynthetic pathway of anandamide was suggested in which anandamide was released from N-arachidonyl-phosphatidylethanolamine by the catalysis of a phospholipase D [24].

3. Organ distribution of anandamide amidohydrolase

Both the hydrolase and synthase activities were screened in various rat organs [25]. Each organ from a Wistar rat was homogenized in 5 times the volume of 20 mM Tris-HCl buffer (pH 8.0) containing 0.32 M sucrose. The homogenate (0.1 mg protein) was assayed for the anandamide hydrolase and synthase activities. As presented in Figure-6, liver showed by far the highest specific activities of hydrolase and synthase (4 - 5 nmol/min/mg protein).

![Figure-6](image)

Figure-6. Distribution of the anandamide hydrolase and synthase activities in rat organs. Native homogenates and acetone-treated homogenates (0.1 mg protein) prepared from various organs of rat were subjected to the enzyme assays. N.D., not detected.
Considerable activities were also detected in cerebrum, cerebellum, testis and parotid gland. In most of the organs tested, the synthase activity was comparable to the hydrolase activity. However, we noted that the synthase activity was much higher than the hydrolase activity in small intestine. Since the homogenate of small intestine inhibited the hydrolase activity of rat liver microsome, we presumed the presence of endogenous inhibitory factors in the small intestine homogenate. The factors were heat-stable and extractable with acetone. The acetone extract inhibited the hydrolase activity dose-dependently, and the synthase activity was less inhibited. We separated the acetone extract by thin-layer chromatography, and scraped the bands of silica gel corresponding to various lipids, separately. The lipids were eluted from each band with methanol, and tested for the inhibitory effects. The results showed that the bands corresponding to free fatty acids, monoacylglycerols and polar lipids inhibited the hydrolase activity. In agreement with this result, when 500 μM of pure free oleic acid, 2-arachidonoyl glycerol, or phosphatidylcholine was added to the reaction mixture, the hydrolase activity of the liver microsome enzyme was reduced to 21%, 28% and 51%, and the synthase activity was reduced to 52%, 63% and 73%, respectively.

In consideration of such endogenous lipid inhibitors, we treated the homogenate of small intestine with 90% cold acetone, and the resultant pellet was resuspended in Tris-HCl buffer. As we expected, the specific hydrolase activity of the homogenate was increased about 10-fold by the acetone precipitation. Then we re-examined the organ distribution of the hydrolase and synthase activities with the acetone-treated homogenates. As shown in Figure-6, small intestine had a high specific activity of the hydrolase (2.0 nmol/min/mg protein), second only to liver. Stomach and colon also showed considerable activities of the hydrolase. Furthermore, we examined distribution of the anandamide amidohydrolase mRNA in rat organs. As analyzed by Northern blotting using 25 μg of total RNA from various organs, an intense band around 2.5 kb was detected in small intestine and stomach as well as liver (Figure-7). Faint bands were observed at the same position in the other organs tested except heart.
Previously, Desarnaud et al. reported that the hydrolase activity of rat was high in liver, brain and testis, and low in small intestine [9]. In this study we demonstrated considerable contents of anandamide amidohydrolase in gastrointestinal tract like small intestine, stomach, and colon. The enzyme in gastrointestinal tract may play a role to detoxify exogenous bioactive fatty acid amides such as anandamide and oleamide. Recently chocolate was reported to contain anandamide and other fatty acid ethanolamides [26].

![Figure-7. Northern blot analysis of the anandamide amidohydrolase mRNA in rat organs.](image)

![Figure-8. Distribution of the anandamide hydrolase and synthase activities in porcine ocular tissues. Homogenates (0.1 mg protein) prepared from various parts of porcine eyes were subjected to the enzyme assays.](image)
Since eye has been known to be a target of cannabinoids [27], and anandamide reduced intraocular pressure and caused conjunctival hyperemia [28,29], we were interested in distribution of anandamide amidohydrolase in ocular tissues. When the homogenates of various tissues of porcine eyes were tested, retina, choroid, iris, optic nerve and lacrimal gland showed high specific activities of the hydrolase and synthase comparable to those in porcine brain (Figure-8) [15]. When subcellular distribution of the enzyme in retina was examined, the enzyme activities were mostly recovered in the particulate fractions rather than in the cytosol in agreement with the brain enzyme. Lens did not show either the hydrolase activity or the synthase activity.

PERSPECTIVES

In the past five years our knowledge on anandamide amidohydrolase was greatly increased. Using recombinant enzymes, catalytic properties of the enzyme will be studied in detail. Physiological significance of the enzyme is also to be elucidated. Potent and specific inhibitors for the enzyme will be good tools to investigate physiological and pathophysiological importance of anandamide.
REFERENCES


BIOSENSORS: NEW TOOLS FOR PHARMACOLOGICAL AND DRUG ANALYSES
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ABSTRACT

Biosensors are analytical devices which combine, in close connection, a physical transducer and a biological element. Typical physical transducers are electrodes (Pt, Au, C, Glass...), optical fibres, piezoelectric elements, thermistors ... and the biological elements are proteins (enzymes, antibodies, peptides ...), nucleic acids (DNA ...), bacteria, tissues and lipids from cell membranes. The unique design of biosensors allows application in wider domains than the classical analytical techniques. Similarly to the latter, biosensors may be used in clinical laboratories for the analysis of physiological parameters such as glucose, lactate, creatine, urea, uric acid... with however the advantage of requiring a minimum sample treatment. This is offered thanks to the elaborated sensing tip of the biosensor which combines, in addition to the biological layer, one or two membranes which protect the biosensor from fouling and which, relatively selectively, extract the analyte of interest from its complex environment. Ideal biosensors are reagentless and smart because auto-calibrated. Over classical analytical methods, biosensors offer improvements in rapidity of the analysis allowing on-site control and quantification of vital parameters such as in hospital emergency situations (surgery, drug intoxication, addiction...).

Thanks to the immobilized biological elements, biosensors can be advantageously applied in drug toxicity/activity test screening. Some biosensors may be applied as detection systems combined to separation techniques (HPLC). Currently efforts are dealing with the development of DNA based sensors for the detection of DNA damage or for the sequence-specific hybridization detection of various viral or bacterial DNA (e.g., HIV-1, TB) and for drug - DNA interaction studies.
Immunosensors are available for on-line monitoring of antigen-antibody reactions and are especially useful in affinity studies and screening of antibodies. New efforts, at a molecular level, are oriented towards the reconstitution of the biological element natural environment at the sensing tip for improved stability and optimum activity/affinity.

**Keywords:** Biosensor, Drug.

**INTRODUCTION**

Analytical techniques for drug analysis have considerably been improved in the last ten years mainly in terms of separation efficiency and detection capabilities. Modern instrumentation offers sensitivity, automation and allows high sample throughput but the equipments are rather elaborated, expensive and generally require well trained operators. Other devices, called biosensors, are under extensive investigation since they may offer alternative ways in pharmacological and drug analyses. Biosensors, as indicated below, can be advantageously applied in industrial and clinical analytical laboratories. Yet, in addition to laboratory instrumentation, there is a demand for decentralized testing. Actually there is a need for portable, simple to use and cost effective detection devices for screening for drugs of abuse (cocaine, opiates...), for real time monitoring in hospital intense care units (anaesthetics, anticoagulants, antitumor drugs...), for routine and emergency assays in physicians' office (salicylates, theophylline, digoxine, neuroleptics, antibiotics...), in non-clinical situation (in sports, employee testing ...) [1]. With this respect, bedside testing of drugs has an obvious appeal to physicians. It allows great reduction for the turn around time for the assay and a better security with respect to activity versus toxicity.

The investigation of therapeutic drugs using biosensors is of great pharmacological interest from a mechanistic point of view. With this respect, the development of biological receptor based transducers can significantly contribute to the understanding of the processes governing drug-receptor interactions. It is well-known that drugs act on either receptors, enzymes or
membranes. The interaction may be more or less biologically specific and is generally potent in very high dilution. High specificity of some drug receptors may be exemplified with drugs existing in two enantiomeric forms, the two isomers exhibiting different activity. By analogy with the enzyme active site, the drug receptor is thought to be a part of a protein structure in a hydrophobic environment (e.g. cholinergic receptors). New strategies consist to mimic living cell membranes by reconstitution of the natural environment of proteins (enzymes, receptors...) onto physical transducers.

**BIOSENSORS**

Biosensors are analytical devices which comprise a biological component intimately connected or integrated with a physical transducer [2] and which can be regarded as attractive tools with respect to the above cited demands. The basic idea in the development of biosensors is to provide a reagentless sensing device allowing selective and sensitive analysis with minimum sample treatment. The concept of a biosensor has been first described in the early sixties, it consisted of the enzyme glucose oxidase immobilised onto a platinum electrode for the determination of glucose. Since then, a variety of biological species and transducers have been considered for biosensor development (Table 1).

**Table 1. Classification of biosensors**

<table>
<thead>
<tr>
<th>Biological element</th>
<th>Transducer</th>
<th>Detection mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme</td>
<td>Electrodes</td>
<td>Amperometry</td>
</tr>
<tr>
<td>Antibody</td>
<td></td>
<td>Potentiometry</td>
</tr>
<tr>
<td>Cell</td>
<td></td>
<td>Impedimetry</td>
</tr>
<tr>
<td>Tissue</td>
<td></td>
<td>Conductivity</td>
</tr>
<tr>
<td>Receptor</td>
<td>Optical fiber</td>
<td>Optical</td>
</tr>
<tr>
<td>Nucleic acid</td>
<td>Surface plasmon device</td>
<td></td>
</tr>
<tr>
<td>Lipid</td>
<td>Thermistor</td>
<td>Thermal</td>
</tr>
<tr>
<td></td>
<td>Quartz cristal</td>
<td>Piezoelectrical</td>
</tr>
</tbody>
</table>
Electrochemical biosensors

Amperometric biosensors have been most investigated because of the inherent sensitivity and selectivity in current monitoring at a constant applied potential and they have been mainly used for the analysis of blood constituents. The market of biosensors in healthcare is dominated by amperometric biosensors for glucose monitoring e.g., in 1995-96 the market of the ExacTech glucose analyzer (see below) of MediSense, which is ranked top one in the field, was $170,106. Considering that more than 5% of the world population suffers from diabetes, it is understandable that constructors have focused their attention to glucose monitoring devices. This was successfully achieved thanks to the remarkable stability of the readily available enzyme glucose oxidase. Yet many other enzymes and more and more antibodies are commercially available leading to the development of biosensors for a variety of physiologically interesting compounds such as lactate, urea, uric acid, creatine, glutamate, etc.

The first generation of biosensors operated by enzymatic action on the analyte to be investigated. Using electrochemical detectors, some product formation (e.g. hydrogen peroxide, o-quinone, H₂, ammonia) or reactant consumption (oxygen) is monitored by either amperometric or potentiometric electrodes.

The second generation of amperometric enzyme electrodes comprise an immobilized artificial redox mediator for enzyme cofactor or coenzyme regeneration and electrochemical signal transduction. Redox mediators are generally small molecules (iron complexes such as ferrocene derivatives) which shuttle the electron from the cofactor (e.g. FAD/FADH₂) to the electrode surface and which exhibit rapid electron transfer at the electrode. The commercial launch of these biosensors [3], leaded by the ExacTech glucose pen, considerably stimulated biosensor research activities. Several enzyme and cell (microbial cell, plant tissue...) immobilized amperometric electrodes (first and second generation) have been investigated as possible sensing devices for drug analysis as illustrated in Table 2. Depending on their
configuration and shape (microsized), these sensors may be used for medical diagnosis as portable instruments or may see some useful applications in the area of industrial process control.

Table 2. Examples of electrodes for drug analysis

<table>
<thead>
<tr>
<th>DRUG</th>
<th>SENSING ELEMENT</th>
<th>CHARACTERISTICS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol, peroxides, antiseptics</td>
<td>GCE/Tyrosinase</td>
<td>Linearity: 0-40μM [5]</td>
</tr>
<tr>
<td>Theophylline</td>
<td>Carbon strip/Alkaline phosphatase</td>
<td>Linearity: 0-300μM [6]</td>
</tr>
<tr>
<td>Acetylsalicylic acid</td>
<td>Carbon strip/aryl acyl amidase</td>
<td>Linearity: 1-7 mM, Stability: 3 months [7]</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>Carbon strip/aryl acyl amidase</td>
<td>Linearity: 0-3 mM, Stability: 6 months [8]</td>
</tr>
<tr>
<td>Theophylline</td>
<td>Pt/Theophylline oxidase + Cytochrome C (FeIII)</td>
<td>Linearity: 0-0.3 mM, DL: 2 μM, Life time: 35 days [10]</td>
</tr>
<tr>
<td>Phenol</td>
<td>TCNQ-graphite/-tyrosinase/phenolase</td>
<td>DL: 0.2 μM [12]</td>
</tr>
<tr>
<td>Glutathione</td>
<td>GCE/Glutathion Peroxidase</td>
<td>Linearity: 0.2-2.4 mM [13]</td>
</tr>
<tr>
<td>Insulin</td>
<td>Pt/anti-Insulin IgG/GA/Protein A/</td>
<td>Linearity: 2-200 μIU [14]</td>
</tr>
<tr>
<td>L-aspartate</td>
<td>Pt/BSA-GA/Aspartate aminotransferase/Glutamate</td>
<td>Linearity: 1μM-1mM, DL: 1 μM [15]</td>
</tr>
</tbody>
</table>
oxidase Life time: 1 month

Opiates

Pt/Heroin esterase/Morphine DL: 23.7 μM [16]

Dehydrogenase

Tissue and Microbial Cells

Catechol [17]

CPE/Eggshell (Polyphenol oxidase) DL: 1 μM Life time: 23 days Linear: 25 μM

Oxygen electrode/Potato slice (polyphenoloxidase) DL: 10 μM

0.2mM[18]

L-Proline [19]

Oxygen electrode/Pseudomonas DL: 10 μM Linear: 0.01 - 1 mM

Phenols [20]

Graphite/Mushroom or Banana Tissue DL: 30 μM Linear: 0 - 5 mM

DL = detection limit, CPE = Carbon paste electrode, GCE = Glassy carbon electrode, TCNQ= Tetracyanoquinodimethane, NMP = N-methylphenazinium, GA = glutaraldehyde, BSA= Bovine serum albumin, HRP = Horseradish peroxidase.

It is worth to point out the considerable interest in the use of tyrosinase in amperometric biosensors. This enzyme develops polyphenol oxidase and catechol oxidase activities and is suitable for the development of biosensors for phenol and catechol derivatives of pharmaceutical interest [4,5,21,22]. Measurements are quite selective since the enzymatic oxidation product (o-quinone) can be detected at a low applied potential (- 0.2 V vs Ag/AgCl) i.e. with minimum risk of interferences by oxidizable species:

\[
\text{phenol} + O_2 \xrightarrow{\text{Tyrosinase}} \text{catechol} \quad (eq.1)
\]

\[
\text{catechol} + O_2 \xrightarrow{\text{Tyrosinase}} \text{o-quinone} + H_2O \quad (eq.2)
\]

\[
\text{o-quinone} \xrightarrow{\text{Electrode}} \text{catechol} \quad (eq.3)
\]
Enzyme electrodes with immobilized tyrosinase or horseradish peroxidase have been shown to be suitable for analyses in organic solvents [23,24]. Many pharmaceutical formulations contain therapeutic drugs or preservatives which are not readily dissolved in aqueous media and such biosensors may be very attractive for quality-control and process monitoring in the pharmaceutical industry.

Amperometric biosensors based on peroxidase (hemoprotein) can be regarded as third generation devices because the regeneration of the enzyme, in the presence of hydrogen peroxide, is directly observed at the carbon electrode surface[25,26]. Peroxidase based electrodes are suitable for hydrogen peroxide determination but also for phenols and aromatic amines and phenothiazines [26-28].

The development of disposable single-use electrochemical biosensors by screen-printing technology is preferred when enzyme reusability is compromised or if there is a risk of mediator leaching out of the immobilized biocatalyst layer [29]. This has been achieved for glucose/ferrocene immobilized carbon strips [3], for theophylline [6], salicylates [7] and acetaminophen [8,9] biosensors.

The immobilization of an antibody onto the electrode surface may be considered as well. This was achieved for the determination of insulin in serum using an antibody immobilized electrode with a competitive immunodetection in the presence of glucose oxidase-labelled insulin [14]. Plant tissues have also been shown to be suitable for biosensor development and the resulting devices exhibit improved stability over the purified enzyme base electrodes [17,18,20].

The monitoring of the neurotransmitter acetylcholine is a considerable challenge in pharmacological analysis. The enzymes acetylcholine esterase and choline esterase may be immobilized in microreactors connected to flow injection systems or to HPLC columns and the liberated hydrogen peroxide detected amperometrically [30] but these enzymes may also be directly immobilized onto the surface of solid electrodes with detection limits of the order of 1 μM [31-34].
Potentiometric pH sensitive electrodes (glass electrode and Ion Selective Field Effect Transistors: ISFETs) have been preferentially used for penicillin determination by immobilizing penicillinase (eq.4) or penicillin amidase (eq.5). The biosensors were comprised in off-line flowing systems allowing penicillin monitoring and its determination in fermentation broths [35].

\[
\text{Penicillin} + H_2O \rightarrow \text{Penicilloate} + H^+ \quad \text{(eq.4)}
\]

\[
\text{R'-CO-NH-R''} + H_2O \rightarrow \text{RCOOH} + H_2N-R'' \quad \text{(eq.5)}
\]

The immobilization of living cells (adsorbed or entrapped in a fibrin clot) has been described for the indirect detection of any metabolism change in cell physiology in the presence of xenobiotics. The sensing system uses a light addressable potentiometric sensor (silicon based device: Si-nitride) to measure the rate at which the immobilized cells acidify their environment [36]. Authors showed that this "silicon microphysiometer" is particularly useful for screening for new receptor ligands, new therapeutic drugs and also for toxicology [37].

The recent development of DNA hybridization biosensors holds great promise for obtaining sequence-specific informations. Electrochemical DNA or Peptide Nucleic Acid biosensors rely on the immobilization of a single-stranded DNA sequence onto solid carbon electrodes for hybridizing with its complementary strand to give rise to voltammetric or potentiometric signals at the ng/ml sensitivity range. [38,39].

**Optical biosensors**

Several optical biosensors using fiber optics for enzyme or antibody immobilization and wave guide have been described for hydrogen peroxide, urea, ammonia, IgG, ATP, ethanol, phenytoin, penicillin etc. [40]. The latter combines penicillinase and a pH sensitive fluorescent dye in a suitable polymer (polyacrylamide) and is applicable in batch conditions and in continuous flow analysis [35].
Other optical sensors exploit surface plasma resonance (SPR) which consist to measure the change in the angle of reflected light of a laser source impinging on a thin gold layer with immobilized reagent (antigen or antibody). The magnitude of the angle change is related to variation in the refractive index at the biosensor surface when the ligand analyte binds to its antibody or receptor [41]. Such unique biosensors allow label-free immunoassay with on-line real time monitoring and screening for new antibody.

Thermal biosensors

Most biological reactions are exothermic and heat sensitive biosensors have been developed for biotechnological processes monitoring and control. The enzyme thermistor is confined in a miniaturized column and it measures, in a differential mode, the reaction heat liberated because of the biocatalytic reaction. Devices for on-line monitoring of penicillin production in bioreactors have been described [42].

Piezoelectric biosensors

A quartz crystal vibrates at a well defined frequency and its vibration is affected by any mass change occurring on its surface following the Sauerbrey equation:

$$\Delta F = -2.3 \times 10^3 F \Delta M / A$$

where $\Delta F$ is the variation of frequency (Hz), $F$ the frequency of the vibrating quartz (MHz), $\Delta M$ the adsorbed mass (g) and $A$ the surface of the quartz ($cm^2$). Provided that care is taken to avoid unspecific adsorption, piezoelectric biosensors are particularly suitable for real time monitoring of protein adsorption and immunoreactions in flow systems and in the gas phase [43]. Other piezoelectric crystals (gold coated) covered by a membrane mimicking layer or by synthetic lipids may serve for the detection of surface active compounds such as surfactants, odors, anaesthetics and antibiotics [44].
REFERENCES

25. Gorton, L., Jonsson-Pettersson, G., Csoregi, E., Johansson, C., Dominguez, E., Marko-Varga,