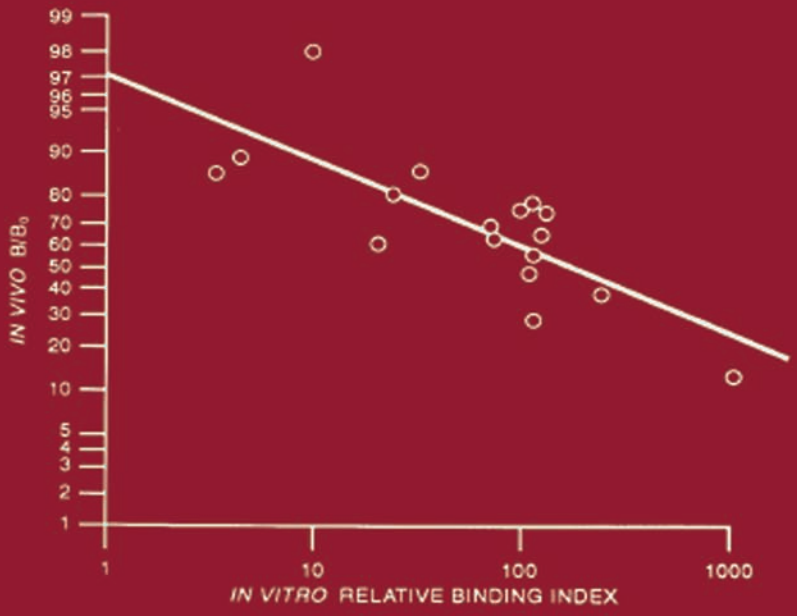
DRUGS AND THE PHARMACEUTICAL SCIENCES VOLUME 55

Radiopharmaceuticals

Chemistry and Pharmacology



edited by
Adrian D. Nunn

Radiopharmaceuticals

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Radiopharmaceuticals

Chemistry and Pharmacology

edited by
Adrian D. Nunn

Bristol-Myers Squibb
Pharmaceutical Research Institute
New Brunswick, New Jersey

Marcel Dekker, Inc. New York • Basel • Hong Kong

ISBN: 0-8247-8624-6

This book is printed on acid-free paper.

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

Current printing (last digit):
10 9 8 7 6 5 4 3 2 1

PRINTED IN THE UNITED STATES OF AMERICA

PREFACE

Nuclear medicine exists in two forms which use slightly different technology to produce images of the in vivo distribution of injected radioactive compounds. For routine clinical use single photon emission tomography (SPECT) predominates and uses mainly Technetium-99m. On the other hand, there is an extensive research effort using positron emission tomography (PET), and here Carbon-11, Fluorine-18 and Oxygen-15 are most used. As yet PET has not been widely used in a clinical setting because the radionuclides it uses are short lived and cyclotron produced.

Clinically, nuclear medicine has been viewed as the Cinderella of medical imaging because it is seen as being 'low tech' (having been around for much longer), is relatively inexpensive to perform, and because the resolution of nuclear imaging devices is inferior to that of the newer CT or MRI machines. This means that morphological images obtained by the latter devices are in almost all cases superior. Radiopharmaceuticals have held a similar position relative to therapeutic drugs because of the dominant position of metal complexes which has isolated the practitioners from the main pharmaceutical chemistry population. Coupled with the scarcity of knowledge of the element most frequently used in radiopharmaceutical chemistry, technetium (a result of current stocks of the element being totally man-made), this has generated a view of radiopharmaceutical chemistry as being something of a 'Black Art' and an imprecise one at that.

In recent years the pressure on nuclear medicine to relinquish morphological imaging to better techniques has fueled attempts to develop functional imaging. Here nuclear medicine has an advantage over the other techniques as the quantities of tracer injected are many orders of magnitude less than those used for CT or MRI and so do not disturb the very processes that are being measured. In addition, the availability of radionuclides of carbon, fluorine and oxygen allows the study of compounds that are well known in conventional pharmaceutical circles. As a result, nuclear medicine has started to exploit its true niche, that of measuring regional function. Along with this has come an evolution, some would say a revolution, in radiopharmaceutical chemistry.

Some ten years ago the routine clinical field, dominated by technetium, concentrated on increasing the delivery of drug to specific tissues. For example, about 50% of the injected dose of phosphonate complexes are taken up by the skeleton within about half an hour after injection and used to depict blood flow which is indicative of new bone deposition around metastases. About 90% of the injected dose of iminodiacetic acid complexes can be excreted by the liver within about the same time to measure the excretory functions of this organ and its associated systems. In recent years attention has concentrated on designing compounds that measure perfusion and here the common targets of the myocardium and the brain have presented much more formidable problems. Needless to say, there are now a variety of technetium compounds that are efficacious for the measurement of perfusion in these tissues and a large body of structure distribution (as opposed to activity) relationships has been constructed. This knowledge includes not only information on the stereo specific interaction of metal complexes with enzyme systems and transport mechanisms, information which is adaptable to compounds other than metal complexes.

In the PET field the blossoming of knowledge of receptor systems has proven irresistible, and much work has been expended in radiolabelling receptor binders and using them to gain quantitative information on receptors in vivo as a function of disease or therapy. To a limited extent this has also occurred in the SPECT field. In many cases this has led to significant interaction between pharmaceutical companies and PET centers in attempts to better understand the pharmacology of new (and old!) therapeutic drugs. In addition, as measurement techniques have improved, the routine analysis of metabolism using PET techniques has occurred. The use of PET techniques is not just an extension of classical drug metabolism and pharmacological techniques; it provides a quantum leap in information. Already the use of such techniques to examine, for instance, receptor occupancy in psychoactive drug therapy is being discussed as are means of diagnosing or staging disease and therapy by measuring receptor density or occupancy.

This book is arranged somewhat along the traditional competitive lines of SPECT vs PET. In the past there has been a great gulf between the clinical usefulness of SPECT agents vs PET and the scientific elegance of PET vs SPECT. This gulf is rapidly closing. The clinical use of PET agents is becoming routine and technetium-based SPECT agents are now as scientifically elegant as their PET counterparts.

The authors were asked to write their chapters in such a way as to provide a review for the practitioners of the disciplines embodied in them yet still provide easily obtainable information to non-practitioners. It is not the intent of this book to list every radioactive compound made and used in vivo and how to make it; rather it is to describe the philosophy behind why one approach is better than another.

In addition, an attempt has been made to present the current state of knowledge such that others may glean as yet unseen information from it. The chapters on technetium agents should displace any misconceptions that abound

that classical pharmacology or drug design is not being embraced in radiopharmaceutical chemistry and should provide useful information for the developers of other inorganic drugs. The precious metals are not the only inorganics to be administered to patients and probably represent a smaller pharmacological base. In addition, the use of many peptide or peptide-like ligands for technetium should provide food for thought for those musing on the in vivo interaction of small peptides with metals. The chapters on PET agents should be attractive to clinical pharmacologists and the like who should be interested and perhaps apprehensive that the fate of their compounds in vivo is now much easier to determine.

Adrian D. Nunn

ACKNOWLEDGEMENTS

The cost of including the color prints in this volume was defrayed by a grant from Squibb Diagnostics, a division of Bristol-Myers Squibb.

I wish to thank Mr. Bruce Kuczynski, Mr. Al Bauer, and Ms. Lee Harrington for their technical assistance in the preparation of this manuscript.

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Development of Positron Emitting Brain Perfusion Agents

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I. INTRODUCTION

The objective of this chapter is to review the development of positron-labeled brain perfusion agents. Particular attention will be given to understanding the physiology involved in the transport of molecules into the brain, identifying the molecular properties of compounds which affect their transport into the brain, and understanding the radiochemical parameters (selection of the radionuclide, labeling position, etc.) which must be considered in the design and synthesis of the radiopharmaceutical. Discussion of the kinetic models and mathematics associated with the measurement of rCBF have been given elsewhere and will not be included in this chapter [1-6].

II. OVERVIEW

The in vivo behavior of brain perfusion agents can be categorized as belonging to one of the following two classes:

1. The agent is freely permeable across the Blood Brain Barrier (BBB) and are thus free to diffuse both into and out of the brain. In this case, blood

flow is evaluated by monitoring the washout of radioactivity from the brain after some degree of tissue saturation is achieved.

2. The agent demonstrates high brain uptake and is subsequently trapped in the brain tissue by one or more mechanisms. In this case, the fixed distribution of radioactivity in the brain is proportional to regional cerebral blood flow (rCBF). It is particularly important here that the retention of the activity in the brain be unrelated to either changing cellular metabolism or tissue pathology, since either one would potentially compromise the validity of the rCBF measurement.

The uptake and clearance of freely diffusible tracers in the brain are dynamic processes and as such they are ideally suited for evaluation by Positron Emission Tomography (PET) since data collection is rapid. (As we will see in subsequent chapters, radiopharmaceuticals evaluated by Single Photon Emission Computerized Tomography (SPECT) require significantly longer residence time in the tissue being imaged due to longer data acquisition times). The ability of a compound to be freely permeable into the brain is based on several anatomical considerations. Exchange occurs between the blood plasma and the extracellular fluid in the capillaries. The capillaries in the brain are structurally different from those in the rest of the body, in that the junctions between endothetial cells are tight, fenestrae are absent, and pinocytic vesicles are rare [7]. Thus in the brain, transcapillary transport of molecules must occur directly through the capillary cell membranes. This structural difference between general and brain capillaries is responsible for the restricted distribution of many polar molecules across the brain capillary network and is the basis of the concept of the blood brain barrier [8].

The overall process by which molecules enter the brain, involves three distinct events.

1. Movement of the molecule from the lumen of the capillary to the surface of the capillary membrane.

2. Transport of the molecule through the capillary membrane.

3. Movement of the molecule away from the capillary membrane and into the brain.

Interference with any one of these processes will adversely affect the uptake of the molecule into the brain.

The major molecular properties of a compound which affects its movement across the BBB are: charge, lipid solubility, extent of protein binding, and molecular size [9,10,11,12,13]. There is also evidence that local dipole moments in the molecule, as well as, the extent of intermolecular hydrogen bonding (e.g. hydrogen bonding of the molecule with plasma water, etc) also reduce the transport of molecules across the BBB [14,15].

A. Extraction vs. Flow

The extraction (E) of a compound by the brain can be related to blood flow (F) by the following equation,

$$E=1-e^{-PS/F} \qquad (1)$$

where P is the permeability of the compound and S is the surface area of the capillary [16]. One can determine the permeability of a compound across the blood brain barrier by monitoring the time-course of the tracer through the brain following its internal carotid artery injection [16,17,21].

A compound is said to be freely permeable across the BBB when it has an extraction value of 1.0, whereas compounds which have limited permeability across the BBB have an extraction value of between 0.0 and 1.0. It can be seen from equation 1 that the extraction value of compounds not freely permeable across the BBB is flow dependent. For example, [^{11}C]methanol has an extraction value of 0.99 at a cerebral blood flow of 31 ml/100g/min, but an extraction value of 0.84 at a cerebral blood flow of 50 ml/100g/min [16]. It should also be apparent from equation 1 that in some instances only elevated

rCBFs will discriminate between compounds which are freely permeable across the BBB and those that are highly permeable across the BBB. For example, [^{11}C]diphenylmethanol has an extraction value of 1.0 at both 46 and 57ml/100g/min but decreases to 0.9 at 70ml/100g/min [18]. A significant underestimation of rCBF can result from data obtained when using a molecule whose extraction value is flow dependent.

B. Selection of the Radionuclide and Its Position in the Molecule

The two most important parameters to consider once the selection of the molecular structure has been determined are the choice of the particular radionuclide and the location of the radionuclide in the molecule. The location of the radiolabel in the molecule often reflects the ease of introducing the radiolabel at a particular position in the molecule. With half-lives as short as a few minutes, it is usually most convenient to introduce the label as late in the synthesis as possible. A comprehensive discussion of the synthetic organic chemistry involved in the preparation of radiolabeled precursors (e.g. $^{11}CH_3I$, $^{11}CH_2O$, etc.) and their use in the preparation of short-lived positron-labeled radiopharmaceuticals is beyond the scope of this chapter and the interested reader is referred to several excellent review articles which address this subject [19,20].

Several positron-emitting rCBF agents used in the clinic are radiolabeled with either O-15 ($t_{1/2}$=123 sec) or C-11 ($t_{1/2}$=20.4 min). Raichle and co-workers advocate the use of O-15 based on practical considerations of patient dosimetry and research protocol [21]. Clinical measurements of rCBF using freely diffusible tracers requires data collection periods of less than 1 minute [21]. Therefore approximately equivalent millicurie doses of the radiopharmaceutical must be administered to the patient regardless of the half-life of the radionuclide [21]. Selection of a radionuclide with a shorter half-

life thus serves to reduce the radiation exposure to the patient as well as reducing the time necessary for background radioactivity (remaining from the prior study) to decay before performing a subsequent rCBF measurement.

C. Metabolic Integrity and Radiochemical Purity of the Radiopharmaceutical

It is all to easy to overlook the possibility of rapid in vivo metabolism of a radiolabeled compound when one is considering the development of any new radiopharmaceutical. Any metabolism which affects the radiolabeled compound, resulting in multiple radiolabeled metabolites, has the potential to complicate or even invalidate the meaningful interpretation of an experiment. Therefore it is most desirable that the radiolabeled molecule remain intact and unmetabolized throughout the duration of the study. Likewise it is only prudent for the radiochemist to ensure that a radiopharmaceutical preparation is free from any radiolabeled impurities which might compromise the experiment.

D. Oxygen-15 Labeled Water

Oxygen-15 labeled water was one of the first tracers suggested for use as a rCBF agent and remains today the most widely used positron-emitting rCBF agent [22]. The advantages of $[^{15}O]H_2O$ are that it is biologically inert, easy to prepare, highly permeable across the BBB, and it has a short half-life. The disadvantages of $[^{15}O]H_2O$ are that it is not **freely** permeable across the BBB (thus its extraction value is flow dependent) and it requires an on-site cyclotron for its production. $[^{15}O]H_2O$ can be conveniently prepared by the exchange reaction between $[^{15}O]CO_2$ and H_2O [23]. Alternatively $[^{15}O]H_2O$ can be prepared <u>in vivo</u> by the inhalation of $[^{15}O]CO_2$. In this procedure $[^{15}O]CO_2$ is immediately converted upon entry into the lung vasculature to $[^{15}O]H_2O$ by

the enzymatic action of carbonic anhydrase located in the red blood cells [24].

$[^{15}O]H_2O$ has been studied extensively as a rCBF agent in both primates and man. As a result of the different means of administration of the radiopharmaceutical (continuous inhalation of $[^{15}O]CO_2$, single-breath inhalation of $[^{15}O]CO_2$, bolus i.v. injection of $[^{15}O]H_2O$, and continuous infusion of $[^{15}O]H_2O$) different mathemathical models are needed to evaluate the perfusion data obtained in these studies. The most throughly studied methods involve the continuous inhalation of $[^{15}O]CO_2$ and bolus i.v. injection of $[^{15}O]H_2O$ [25,26].

The earliest method of measuring rCBF with $[^{15}O]H_2O$ was the steady-state inhalation technique. In this procedure the patient was required to breath the $[^{15}O]CO_2$ for approximately 10 minutes. At which point an equilibrium was reached between the washout of activity from the brain, the physical decay of the radioisotope and the amount of activity entering the brain through the arterial system. As a result of the equilibrium this activity in the brain remains constant and the rCBF can be calculated [27]. Jones and co-workers have subsequently modified this approach by replacing the continuous inhalation of $[^{15}O]CO_2$ with a constant infusion of $[^{15}O]H_2O$ [28].

Another method of measuring rCBF with $[^{15}O]H_2O$ involves the administration of an i.v. bolus of $[^{15}O]H_2O$ [5,26]. In this method one measures rCBF by monitoring the washout of activity from the brain following the administration of the tracer. The relative advantages and disadvantages of these two techniques are given in Table 1. A more thorough discussion of the relative merits of the different methods of administration of $[^{15}O]H_2O$ is beyond the scope of this chapter and the reader is referred to an excellent review by Ter-Pogossian and Herscovitch [22].

Table 1. Comparison of the Relative Advantages and Disadvantages of $[^{15}O]H_2O$ Prepared by Different Techniques to Evaluate rCBF in Man.

Continuous $C^{15}O_2$ Inhalation	Bolus Intravenous Injection of $H_2{}^{15}O$
Advantages	Advantages
Well-characterized.	Well characterized.
Rapid sampling of arterial blood at specific times not required.	Extensive use.
Suited to single-ring tomographs that operate accurately at low count rates.	Validated by independent method.
First method developed to measure rCBF with $H_2{}^{15}O$.	Excellent agreement between rCBF (true) vs. rCBF (PET).
Sterile, and reliable preparation.	Linear relationship between rCBF and measured radioactivity concentration.
	Well suited to study functional-anatomical correlations within brain.
	Sterile, and reliable preparation

Continuous $C^{15}O_2$ Inhalation	Bolus Intravenous Injection of $H_2{}^{15}O$
Disadvantages	Disadvantages
Non linear relationship between rCBF and measured radiotracer concentration.	Requires rapid data acquisition.
Limited accuracy in heterogenous tissue regions	Requires accurately timed sampling of arterial blood.
Underestimates regions of high rCBF.	Tomograph must be accurate at high count rates.
Requires 10 min. to achieve steady-state.	
Requires continuous and steady production of $C^{15}O_2$ from cyclotron.	
Requires patient participation.	
$H_2{}^{15}O$ not freely permeable across BBB and underestimates regions of high flow.	$H_2{}^{15}O$ not freely permeable across BBB and underestimates regions of high flow.

As previously mentioned the most significant disadvantage in the use of $[^{15}O]H_2O$ is the fact that H_2O is not freely permeable across the BBB [17]. Eichling and co-workers showed that $[^{15}O]H_2O$ is not freely permeable across the BBB in primates when the rCBFs is greater than 30ml/min/100 g [17]. Thus measurements of rCBF in areas of high blood flow (gray matter) will be somewhat less accurate than similar measurements of rCBF in areas of lower blood flow (white matter). However, most clinical studies do not compare gray to white flows, rather, they compare different flows within the gray matter. Although $[^{15}O]H_2O$ is not the "ideal rCBF agent" as a result of its restricted permeability across the BBB at elevated rCBFs, the ease and reliability of its production coupled with the short half-life of ^{15}O have resulted in its widespread clinical use.

E. Alcohols

In an effort to develop a tracer freely permeable across the BBB, Raichle and co-workers prepared a variety of $[^{11}C]$alcohols (methanol, ethanol and isopropanol) and evaluated their permeability across the BBB [16]. While none of these alcohols were freely permeable across the BBB each showed improved permeability compared to that of $[^{15}O]H_2O$ [16]. They also found that the permeability of the alcohol increased with its lipophilicity [16]. They subsequently prepared $[^{11}C]$-1-butanol and found that this tracer was freely permeable across the BBB in baboons at rCBFs of at least 170 ml/min/100g [16]. (An rCBF of 170 ml/min/100g is well above any rCBF which would be encountered in the clinic). As a result of these studies, $[^{11}C]$-1-butanol has received considerable attention as a positron-emitting rCBF agent [29]. Herscovitch and co-workers recently reported a study where paired rCBF measurements were made with $[^{15}O]H_2O$ and $[^{11}C]$-1-butanol in normal human subjects [30]. They observed that the ratio of regional gray matter

(high flow) to white matter (low flow) was statistically different with [^{11}C]-1-butanol than with [^{15}O]H_2O (2.28 vs. 2.01) [30]. These results are consistent with the known limited permeability of [^{15}O]H_2O at high rCBFs [29].

The most convenient preparation of ^{11}C-butanol involves the lithium aluminum hydride reduction of [1-^{11}C]butanoic acid prepared from n-propyl magnesium bromide and [^{11}C]CO_2 [16]. Brodack and co-workers have recently reported the use of robotics for the routine preparation (including purification) of [^{11}C]-1-butanol [31]. This development is particularly noteworthy in that multiple sequential preparations of [^{11}C]-1-butanol can now be prepared with minimal radiation exposure to technical personnel. It should be of no surprise that given the excellent brain extraction of [1-^{11}C]-1-butanol, and the preference for ^{15}O over ^{11}C in rCBF measurement, that [^{15}O]-1-butanol has recently been prepared [32,33,34]. Takahasmi and co-workers have reported the preparation of [^{15}O]-1-butanol suitable for clinical use in a synthesis and purification of 3 minutes via the reaction of [^{15}O]O_2 and tributylborane [34]. The resulting borane complex is then hydrolzyed and the [^{15}O]-1-butanol purified via sequential C-18 Sep-Pak and anion exchange chromatography. The development of a similar robotic system for the routine preparation of ^{15}O-1- butanol would greatly assist in the widespread clinical usefulness of this agent.

F. Lipophilicity

We have already discussed how the extraction of a compound by the brain can be increased by increasing its lipophilicity. However, what effect does continually increasing the lipophilicity of a molecule have on brain extraction? Dischino and co-workers studied the brain extraction of a variety of [^{11}C]-labeled ethers, and alcohols with log $P_{(oct)}$ (partition coefficient for octanol/water) values between -0.7 and greater than 4.0 [18]. They found that a parabolic structure distribution relationship exists between the lipophilicity

of a compound and the fraction of the compound penetrating the BBB during a single capillary transit in adult baboons (Figure 1) [18]. Compounds with log P(octanol) values between 0.9 and 2.5 were found to be freely permeable across the BBB. (Thus any of these compounds could be used instead of 1-butanol for the measurement of rCBF). The apparently decreased extraction of the more lipophilic compounds was shown to be related to binding of the radiolabeled molecules to blood macromolecules [18]. Hansch and co-workers had earlier proposed that a parabolic relationship existed between the lipophilicity of a molecule and its uptake by the brain [35]. (They did not specifically measure brain uptake of the compounds but inferred it from the experiment). They also proposed that the ideal lipophilicity (P_0) of a molecule for passive penetration into the CNS was log P_0=2.0 +0.3 [35]. They also showed that the binding of a wide variety of neutral organic compounds of miscellaneous structure to both bovine serum albumin and bovine hemoglobin appeared to depend entirely on hydrophobic bonding and thus was linearly related to lipophilicity [12].

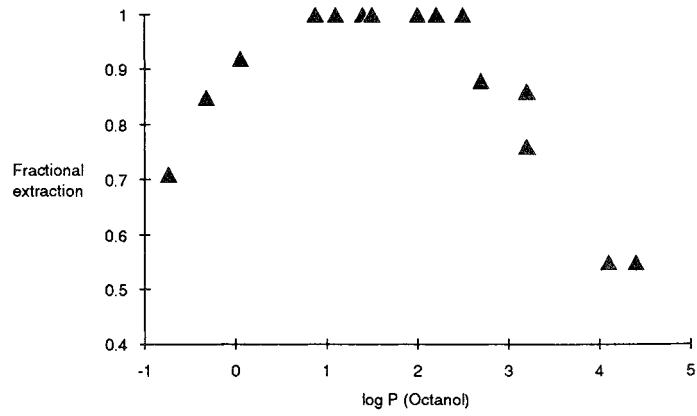


Figure 1. Calculated Extraction of Several C-11-Labeled Compounds Plotted Against Log P (Octanol) Values at CBF of 100 mL min^{-1} hg^{-1} [18].

Returning for a moment to our model describing the overall process by which molecules enter the brain, we can see that the hydrophilic alcohols are most likely restricted in their ability to diffuse through the lipid bilayer of the capillary membranes, whereas, the lipophilic compounds may be unavailable for transport across the membrane as a result of being tightly associated with plasma proteins or that their rate of diffusion from the lumen of the capillary to the capillary wall may be hindered by interactions with plasma proteins, red blood cells, etc.

G. Volatile Agents

Only a few positron-emitting volatile radiopharmaceuticals have been prepared and evaluated for use as rCBF agents. These agents are inert gases and are freely permeable across the BBB. They are not metabolized in vivo and are expired from the body. An important parameter to evaluate in the selection of a potential volatile agent as a rCBF agent is its solubility in both blood and brain tissue. This is most conveniently expressed by the Ostwald solubility coefficient (partition coefficient of a gas between equal volumes of two phases when the phases are in equilibrium) of the gas. When the solubility of the gas in blood is low, the transfer of gas from the alveolar air into the pulmonary blood is low. This results in only low levels of the gas being available for general circulation in the body.

The first positron-emitting inert gas evaluated in man as a potential rCBF agent was ^{77}Kr ($t_{1/2}$=1.2 h) [37]. The low solubility of this gas in both blood and brain tissue, greatly limited the amount of radioactivity being delivered to the brain, and thus it was not possible to obtain high quality images of the brain [36]. Other radiolabeled gases including [^{13}N]N_2O, [^{15}O]N_2O, and [^{11}C]HCCH which are more soluble in blood than ^{77}Kr, have been prepared and evaluated as potential rCBF agents [38,39,40]. However only preliminary reports have been published addressing the synthesis and biological studies of

these gases, and the lack of continued development of these agents might reflect the difficulties associated with either the preparation or purification of these radiolabeled compounds.

The fluoroalkanes have received considerable attention as potential rCBF agents [40,41,42]. $[^{18}F]CH_3F$ and other fluoroalkanes ($[^{18}F]CH_3CH_2F$, $[^{18}F]CH_3CH_2CH_2F$ and $[^{18}F](CH_3)_2CH_2F$) have been prepared by the nucleophilic displacement of iodine by $[^{18}F]F^-$ of the appropriate iodoalkane [41]. These fluoroalkanes are significantly more soluble in blood than ^{77}Kr. Table 2 compares the solubility of these fluoroalkanes in blood to other radiolabeled gases suggested as potential rCBF agents [41]. Whole-body washout kinetics of the $[^{18}F]$fluoroalkanes were conducted in rat to determine if the fluoroalkanes were being metabolized in vivo. Metabolites such as $[^{18}F]F^-$ would not be expected to be expired and might be taken up by the bone or excreted in the urine. $[^{18}F]CH_3F$ was completely cleared from the body within 1 h post-injection, while the other compounds (being more lipophilic) showed much slower clearance from the body. $[^{18}F]CH_3F$ has subsequently been shown to provide accurate measurements of rCBF in man [43]. This work encouraged Stone-Elander and co-workers to prepare $[^{11}C]CH_3F$ from $[^{11}C]CH_3I$ and tetrabutylammonium fluoride [44]. $[^{11}C]CH_3F$ is presently being used in the clinic and the shorter half-life of ^{11}C permits multiple rCBF measurements to be performed in the same individual every 30-40 minutes [44]. Mulholland and co-workers have recently prepared $[^{17}F]CH_3F$ ($t_{1/2}$=65 sec) [45]. The rapid decay of ^{17}F would permit one to obtain sequential rCBF measurements in man every 6 minutes as compared to every 12 minutes as is the case for $[^{15}O]H_2O$. Although only preliminary reports are available on this $[^{17}F]$-radiopharmaceutical, its future appears promising.

Table 2. Ostwald Solubility Coefficients of Selected Gases in Blood at 37°C

GAS	OSTWALD SOLUBILITY COEFFICIENT
$^{11}CH_4$	0.03
^{77}Kr	0.09
^{133}Xe	0.14
$^{13}N_2O$	0.47
$^{11}C_2H_2$	0.90
$[^{18}F]CH_3F$	1.07
$[^{18}F]CH_3CH_2F$	1.22
$[^{18}F]CH_3CH_2CHF$	1.14

H. Antipyrines

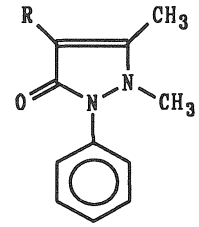
$[^{131}I]CF_3I$ was originally used for the autoradiographic determination of rCBF in animals based on the uptake of a chemically inert, diffusible radioactive tracer [46]. $[^{14}C]$-Antipyrine was later suggested as an alternative agent in rCBF measurements to avoid the technical problems associated with the use of a volatile tracer [47]. Eckelman and co-workers subsequently demonstrated that $[^{14}C]$antipyrine (log P oct = 0.57) was not freely permeable across the BBB [48]. In their study they compared the rCBF values obtained from either $[^{14}C]$antipyrine or $[^{131}I]CF_3I$ in cats, and demonstrated that consistently lower values of rCBF were obtained with $[^{14}C]$antipyrine [48]. Sakurada and co-workers then demonstrated that a more lipophilic derivative of antipyrine, namely 4-iodoantipyrine (log P oct = 1.7) was freely permeable

across the BBB [49]. [^{14}C]-4-Iodoantipyrine is now a commonly used tracer for the autoradiographic determination of rCBF in laboratory animals. Efforts to develop [^{11}C]iodoantipyrine for use as a potential rCBF agent in man were a result of widespread use of [^{14}C]iodoantipyrine in animal studies.

[^{11}C]-4-iodoantipyrine was originally prepared by Campbell and co-workers via the iodination of [^{11}C]antipyrine, prepared from [^{11}C]CH_3I and 3-methyl-1-phenyl-2-pyrazolin-5-one [50]. This preparation suffers from a long synthesis and purification time (65 minutes) thus limiting the amount of radiolabeled material available for biological studies. An alternate synthetic pathway exist for the synthesis of [^{11}C]-4-iodoantipyrine, namely, the methylation of 4-iodo-3-methyl-1-phenyl-2-pyrazolin-5-one with [^{11}C]CH_3I, however this approach is not practical in light of the inherent instability of the precursor under the reaction conditions required for alkylation [50]. Thus although the molecule demonstrates suitable in vivo characteristics to merit use as a rCBF agent, the difficulty in preparing this radiolabel agent in large quantities has greatly limited its clinical usefulness.

Three positron-emitting radiolabeled derivatives of antipyrine have been suggested for use a potential rCBF agents in man: [^{18}F]-4-fluoroantipyrine, [^{11}C]-4-isopropylantipyrine and [^{76}Br]-4-bromoantipyrine. Shiue and co-workers prepared [^{18}F]-4- fluoroantipyrine from antipyrine and [^{18}F]F_2, and recently Diksic and co-workers have reported improved methods for the synthesis and purification of this radiopharmaceutical [51,52]. The replacement of the iodine atom in 4-iodoantipyrine with fluorine will significantly reduce the lipophilicity of the molecule (Table 3). First pass extraction studies of [^{18}F]-4-fluoroantipyrine in cats at high CBFs have shown that [^{18}F]-4-fluoroantipyrine has a higher brain extraction than [^{15}O]H_2O [53]. However the relatively long half-life of fluorine-18 ($t_{1/2}$=109.8 min) would significantly delay multiple rCBF measurements.

Table 3. Partition Coefficients of Selected 4-Substituted Antipyrines.



R	Log P
H	0.57
F	0.72
Br	0.99
I	1.70
$CH(CH_3)_2$	2.15

Stone-Elander and co-workers prepared [^{11}C]-4-isopropylantipyrine from [^{11}C]CH_3I and 4-isopropyl-3-methyl-1- phenylpyrazol-5-one in a synthesis and purification time of 40 minutes and in a radiochemical yield of 40-50% [54]. In contrast to 4-fluoroantipyrine, the replacement of the iodine on 4-iodoantipyrine with an isopropyl group will result in [^{11}C]-4-isopropylantipyrine being slightly more lipophilic than 4-iodoantipyrine. Initial rCBF studies with [^{11}C]-4-isopropylantipyrine in rats at high CBFs indicate that the compound behaves similarly to 4-iodoantipyrine. Lambrecht and co-workers have suggested that [^{78}Br]-4-bromoantipyrine (^{78}Br $t_{1/2}$=6.5 min) be considered as a potential rCBF agent in man [55]. The benefits of using a radionuclide with a shorter half- life in rCBF measurements have been previously discussed. Additionally 4-bromoantipyrine (log P oct = 0.99) is slightly more lipophilic than 4-fluoroantipyrine and thus should demonstrate improved brain extraction as compared to its fluorinated analog.

Thus far we have reviewed the development of freely-diffusible positron-emitting radiopharmaceuticals designed to measuring rCBF in man. All of these agents are highly permeable across the BBB and their transport into the brain is by simple diffusion and not by energy requiring processes (active or

facilitated transport). All of the clinically useful compounds are neutral and with the exception of $[^{15}O]H_2O$, have log P octanol values ranging from 0.9 to 2.5.

There has been only limited research in the development of a positron-emitting rCBF agents which are retained (or trapped due to metabolism) in the brain. $[^{13}N]NH_3$ ($t_{1/2}$=9.96 min) was the first positron-emitting agent of this type to be investigated. Several different investigators soon reported that the transport of $[^{13}N]NH_3$ across the BBB was a function of rCBF, blood pH, as well as the integrity of the blood brain barrier. The effect of blood pH on the equilibrium, $[^{13}N]NH_4^+ + H_2O$ $[^{13}N]NH_3 + H_3O^+$ manifests itself by altering the relative amount of the $[^{13}N]N$- labeled ammonium cation, whose transport across the BBB is greatly restricted [56,57,58,59]. Interest in this agent as an rCBF agent has diminished as a result of its poor extraction across the BBB.

Microspheres are useful in detecting perfusion to an organ since they are trapped in the first capillary bed that they encounter after administration. Although more frequently used to evaluate myocardial perfusion, $[^{11}C]$ microspheres have been used to evaluate rCBF in man [59]. $[^{11}C]$Methylalbumin microspheres and $[^{11}C]$methyl albumin are stable in vivo and can be prepared by the reaction of human serum albumin/human serum albumin microspheres with $[^{11}C]$methyl iodide in useful quantities in approximately 30 minutes [60].

Kizuka and co-workers have prepared N-$[^{11}C$-methyl] chlorphentermine as a potential rCBF agents (Figure 2) [62]. The uptake and retention of this compound is reminescent of that observed with $[^{123}I]$ N-isopropyl-p-iodoamphetamine in that although the compound is not freely permeable across the BBB, the activity in the brain remains constant over 30 minutes. Additional studies to elucidate the mechanism of retention of N- $[^{11}C$-

methyl]chlorphentermine will be necessary if this agent is to be used to evaluate rCBF in man.

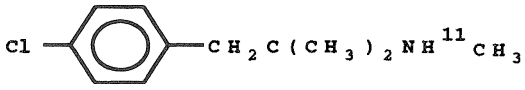


Figure 2. Chemical Structure of N-[^{11}C-methyl]chlorophentermine ([^{11}C]NMCP).

III. GENERATOR-PRODUCED RADIONUCLIDES USED IN THE DEVELOPMENT OF POSITRON-EMITTING rCBF AGENTS

Cyclotrons provide a reliable source of positron-emitting radionuclides, and often, they provide the only practical source of large quantities of these radionuclides. However the operation and maintenance of a cyclotron facility requires a large support staff and represents a major capital investment. Generators also provide a reliable source of radionuclides at greatly reduced expense, however the variety of radionuclides available from generator systems is very limited. The next section of this chapter will address the development of rCBF agents based on four of these systems (^{68}Ge-^{68}Ga, ^{62}Zn-^{62}Cu, ^{82}Sr-^{82}Rb, and ^{122}Xe-^{122}I) [Table 4].

Table 4. Physical Properties of Selected Generator Systems.

DAUGHTER	HALF-LIFE	DECAY MODE	PARENT	HALF-LIFE
Cu-62	9.74 min	B^+ (98%), EC	Zn-62	9.1h
Ga-68	6.81 min	B^+ (90), EC(10)	Ge-68	275d
Rb-82	75 sec	B^+ (96), EC(4)	Sr-82	25d
I-122	3.6 min	B^+ (100)	Xe-122	20.1h

A. Gallium

The development of the ^{68}Ge/^{68}Ga generator system dates back to the 1960's [63,64]. However the development of new gallium-68 ($t_{1/2}$=68 min)

radiopharmaceuticals has been slow due to technical problems associated with the earlier generators and the fact that gallium forms a very strong chelate with the plasma protein transferrin (log K_1=20.3) [65].

The former problem has been effectively resolved by the development of a tin dioxide/ 1 N HCl generator which provides a sterile solution of ^{68}Ga in high recovery yields (75-80%). The ^{68}Ga, in ionic form, is thus ready for immediate use in the synthesis of ^{68}Ga-labeled radiopharmaceuticals [66]. Earlier generator systems (e.g. alumina/ 5 mM EDTA) provided the ^{68}Ga in complexed form ($Ga(EDTA)^{-1}$) [64]. The recovery process was lengthy (30 minutes) and an additional dechelation step was often necessary. The high stability constant of Ga-transferrin, and the large amount of transferrin in human plasma (0.25 mg/100 ml), has required the development of polydentate chelating ligands capable of forming very strong complexes with gallium. Weak chelates of gallium are unstable in vivo and lose their metal to transferrin, (and thus the biodistribution of weak gallium chelates is similar to that of ^{68}Ga transferrin).

Green and co-workers have prepared a variety of gallium tris(salicylaldimine) complexes [67]. The biodistribution of these complexes is significantly different from that of gallium-transferrin. These complexes were well characterized and single-crystal x-ray structural analysis demonstrated that the $(5\text{-MeO-sal})_3$TAME ligands afford a neutral Ga(III) complex by bonding through the three imino-nitrogen lone pairs and the three deprotonated phenolic oxygen atoms [68]. The behavior of these complexes on paper electrophoresis further confirmed that the complexes were neutral. However even though the complexes were neutral and lipophilic (log P (octanol/saline pH 5.2) values ranged from 1.6 to 2.7), none of the compounds showed any brain uptake. In attempting to rationalize the poor brain uptake of these compounds, the authors draw attention to the molecular weight (appr. 600 daltons) of these compounds and suggest that this may be a contributing factor

in the poor brain uptake of the complexes [68].

Levin had previously reported a study where the rat brain capillary permeability was determined for 27 different organic compounds [11]. He showed that the brain permeability, for compounds with a molecular weight of less than 400, could be predicted based on considerations of the lipophilicity of the molecule. Hansch subsequently recalculated Levin's data, and concluded that the penetration of a compound into the CNS is not significantly inhibited by molecular size of the compound, when the molecular weight of the compound is less than 500 [13]. It is important to appreciate here that it is the molecular volume of the compound and not the molecular weight of the compound which affects the transport and uptake of molecules into the brain. This is particularly important in light of current interest in developing both gallium and technetium radiopharmaceuticals. Consider for example the difference in molecular volume between ^{111}In ($<1A^{o}$) and 1-heptanol ($>8A^{o}$). Both ^{111}In and 1-heptanol have similar molecular weights (M. Wt. 116) but there is more than an eight fold difference in their molecular volume [69]. This difference becomes even more striking when one compares the molecular volume of certain metal chelates (e.g. Ga[(5-Meosal)$_3$TAME, M. Wt. 585), where the metal ion is chelated to a polydentate ligand, to other purely organic compounds of equal molecular weight. The reader may find the use of CPK precision space filling models particularly helpful in further understanding the relationship between molecular volume and molecular weight.

How then does the molecular volume of a molecule affect its transport across the BBB? To understand this we must return to our model describing the processes involved in the transport of molecules across the BBB. If the molecular volume of the compound significantly inhibits the rate of diffusion of the molecule from the lumen of the capillary to the capillary membrane, it can be argued that during a single capillary transit, the molecule is unavailable

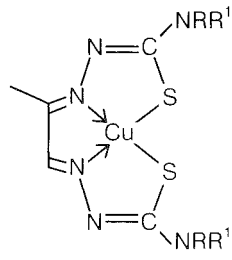
for transport across the BBB since it never comes in contact with the capillary membrane. Additionally the volume of the molecule may be important in the transport of a molecule across the BBB depending on the nature of the mechanism by which the molecule migrates through the lipid bilayer of the cell membrane. As one can see from these arguments, the molecular shape, as well as, the molecular size may be important determinants in evaluating the penetration of compounds across the BBB.

Helmer and co-workers have shown that the binding of a wide variety of neutral organic compounds of miscellaneous structure to bovine serum albumin and bovine hemoglobin is correlated to the lipophilicity of the compounds [70]. However does this relationship between lipophilicity and protein binding apply to metal chelates as well as neutral organic compounds? How does the molecular structure or local dipole moments in the metal chelates affect their association with macromolecules? Moerlein and co-workers have reported that the gallium-LICAM complexes bind to high molecular weight plasma proteins independent of the lipophilicity of the complex [71]. Until the parameters which affect the binding of metal chelates and blood macromolecules are understood, careful studies must be done to prove that the metal chelate is available for transport across the BBB and not tightly associated with blood macromolecules and thus unavailable for transport across the BBB.

B. Copper

^{62}Cu ($t_{1/2}$=9.73 min) is another generator-produced positron- emitting radionuclide which could be utilized to label compounds for rCBF measurements. Green has recently reported some initial studies on a series of neutral lipophilic ^{67}Cu complexes of pyruvaldehyde bis(thiosemicarbazone) derivatives (Table 5) [72].

Table 5. Partition Coefficients of Selected Copper Complexes of Pyruvaldehyde bis (N4-Substituted thiosemicarbazone).



R,R'	Log P
H,H	0.75
CH_3,H	1.97
CH_3,CH_3	2.70

Biodistribution studies of the ^{67}Cu complexes of pyruvaldehyde bis(thiosemicarbazone (PTS), pyruvaldehyde bis(N^4-methylthiosemicarbazone) (PTSM), and pyruvaldehyde bis(N^4-dimethylthiosemicarbazone) ($PTSM_2$) in rats indicate that the latter two compounds show good brain uptake [72]. The two tracers differ in that the maximal brain uptake of the monomethyl derivative remains constant for up to 2h post-injection whereas the dimethyl derivative shows a progressive decline during the first 15 minutes following the administration of Cu-$PTSM_2$ [72]. Although the mechanism by which retention of Cu-PTSM in the brain occurs is not fully understood, there is evidence to suggest that there is a some type of reaction occurring between the copper complex and intracellular sulfhydryl groups [72].

Cerebral extraction studies of CuPTSM in baboons were conducted at CBF of 60 and 90 ml/(min·100g). Extraction fractions of 0.77 and 0.64 were observed at these two flows. These extraction values are less than would have been expected for an organic alcohol or ether with similar lipophilicities and

the measured [PS] product for ^{67}Cu-PTSM was approximately 28% lower than that of [^{15}O]H_2O. Thus although this copper complex is not freely permeable across the BBB, this work is significant in that it identifies another family of inorganic complexes worthy of further development.

C. Iodine

The ^{122}Xe/^{122}I generator systems is unique among generator systems in that the daughter radionuclide can be incorporated into organic molecules via organic bonds [74]. The resulting ^{122}I radiopharmaceuticals are thus expected to differ in their in vivo characteristics from existing generator produced radiopharmaceuticals which are inorganic cations or coordination compounds [74]. The rapid decay rate of ^{122}I ($t_{1/2}$=3.6 min) places strict constraints on the time available for the synthesis and purification of ^{122}I radiopharmaceuticals. Therefore the initial work involved in the development of ^{122}I radiopharmaceuticals has involved developing rapid and efficient radiolabeling procedures [74].

Mathis and co-workers reported the synthesis and purification of a variety of ^{122}I-labeled radiopharmaceuticals, which have been previously radiolabeled with either ^{123}I, ^{125}I or ^{131}I [75,76,77]. Dynamic brain uptake studies of ^{122}I-labeled 2,4-dimethoxy-N,N-dimethyl-5-[^{122}]iodophenylisopropylamine in dogs showed that there was a rapid uptake of activity by the brain which remained essentially constant from 2-10 minutes post- injection. Moerlein and co-workers have also prepared ^{122}I-iodoperidol via the electrophilic iododestannylation of 4-[4- [4-(trimethylstannyl)phenyl]-4-hydroxypiperidino]-4'-fluorobutyrophenone in a synthesis and purification of 5 minutes [78]. Autoradiographic experiments with ^{131}I-iodoperidol in rats have shown that although the compound is not freely permeable across the BBB it shows good cerebral uptake (1.9% ID/whole brain). Cerebral uptake

studies of ^{122}I-iodoperidol in dog have shown that following uptake of the compound by the brain, the activity in the brain remained relatively constant over the first 20 minutes post-injection [78]. The prolonged retention of activity in the brain following the administration of the ^{122}I radiopharmaceuticals indicate their potential use in rCBF measurements as microsphere analogues.

It might also be possible to prepare an [^{122}I] radiopharmaceutical freely permeable across the BBB.

The short half-life of ^{122}I would thus minimize radiation exposure to the patient while also providing for rapid sequential measurements of rCBF. Moerlein has reported the preparation of a variety of neutral lipophilic ^{122}I-labeled aromatic compounds including ^{122}I-labeled iodobenzene (log P= 3.27), ^{122}I- iodoanisole (log P = 3.32), ^{122}I-p-iodofluorobenzene (log P = 3.40) and p-iodotrifluoromethyl- benzene (log P = 4.15) [74]. Although each of these compounds appears to be too lipophilic to be completely extracted by the brain, a less lipophilic iodinated aromatic compound such as p-iodobenzyl alcohol (log P = 2.23) might be a molecule worthy of future consideration.

A major obstacle to the future development of clinically useful ^{122}I or ^{62}Cu labeled radiopharmaceuticals lies in the efficient production and distribution of these generator systems. This will be a very difficult problem to overcome in light of the relatively short half-life of the parent radionculides (20.1h and 9.1h respectively).

D. Rubidium

^{82}Rb available from the ^{82}Sr/^{82}Rb generator system has been used in myocardial perfusion studies and in studies to evaluate the integrity of the BBB [79]. Unlike either ^{122}I or ^{62}Cu, ^{82}Rb is the daughter of a relatively long-lived radionuclide (^{82}Sr $t_{1/2}$=25 day) and a commercial ^{82}Sr/^{82}Rb

generator is available. ^{82}Rb is a monovalent cation and is thus excluded from the brain by the blood brain barrier [79]. The development of a ^{82}Rb labeled freely diffusible tracer or microsphere analog could be very useful. However the extremely short half-life of ^{82}Rb (75 sec) represents a significant hurdle to the radiochemist who must synthesis and purify the ^{82}Rb chelate.

IV. FUTURE TRENDS IN THE DEVELOPMENT OF POSITRON-EMITTING CEREBRAL PERFUSION AGENTS

Significant progress has been made in the past decade in the development of new positron-emitting radiopharmaceuticals for rCBF measurements, as well as for evaluating cerebral metabolism, mapping neuroreceptors, imaging breast tumors, etc. PET is no longer viewed as "a research tool only" and it will soon be used in the routine management of patients. In the future we may observe the development of cyclotron-free PET imaging centers. These institutions will need to be supplied with long-lived positron-labeled metabolic tracers (e.g. ^{18}F-fluorodeoxyglucose) from regional production/distribution centers while utilizing appropriate in-house generators to produce short-lived positron-emitting radionuclides for use in perfusion studies [80]. Additionally as the clinical utilization of PET becomes more widespread, many of the larger medical centers may have several tomographs. This will enable more patients to benefit from PET technology but it will also place increased demands on the both the cyclotron and its staff. Patient output may be improved if the cyclotron can be dedicated to the sole production of the longer-lived radionuclides used to label metabolic tracers (e.g. ^{18}F-labeled fluoro deoxyglucose) while performing perfusion studies with generator produced radionuclides. However $[^{15}O]H_2O$ has served us very well for the past several years and the ease and reliability of its production will guarantee its use as an rCBF agent in the next decade.

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Acknowledgments

The author is particularly indebted to Dr. Adrian Nunn for his continual guidance, suggestions and patience throughout the writing of this manuscript.

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Technetium-based Brain Perfusion Agents

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I INTRODUCTION

In 1978 Oldendorf [1] first suggested the need for new radiopharmaceuticals labelled with Tc-99m, which were capable of providing information on regional cerebral blood flow (rCBF). He perceived that there would be a need for routine evaluation of this parameter of brain physiology, not provided for by transmission computerised tomography (CT), or earlier technetium brain scanning, which only demonstrated blood brain barrier (BBB) defects.

Developments in positron emission tomography (PET) in the late 1970's and iodine-123 radiopharmaceuticals for single photon emission computerised tomography (SPECT) in the early 1980's greatly enhanced the general appreciation for the potential of cerebral perfusion imaging in the diagnosis and management of cerebral vascular disease (CVD) and other neurological disorders. In particular this technique appears well suited to the early assessment of cerebral infarction, in evaluating reversible cerebral ischaemia and the study of epilepsy and dementias [2].

While the development of new positron and I-123 radiopharmaceuticals (reviewed elsewhere in this volume [3,4]) demonstrated the potential of cerebral perfusion imaging, not one is amenable for routine use. The very short half life of most positron isotopes necessitates their production and use at a single location, involving high cost facilities, justifiable at relatively few specialist centres. Iodine-123 suffers from high production costs and limited availability. Tc-99m has none of these disadvantages; it is cheap, readily available, and has near ideal physical properties for use with single photon imaging equipment. As Oldendorf implied [1] it is the preferred isotope for a brain perfusion radiopharmaceutical.

Several investigators have sought to develop a Tc-99m radiopharmaceutical capable of providing an image of cerebral perfusion. This

chapter will examine the progress made from the start of that endeavour in the mid-1970s, to the present.

II MOLECULAR REQUIREMENTS FOR CEREBRAL PERFUSION IMAGING

In order to provide an image of cerebral perfusion, a radioactive tracer must, following intravenous administration, be extracted from the blood stream into cerebral tissue. This extraction process should proceed with high efficiency, and result in a regional distribution of tracer which reflects regional blood flow. In addition, the relative distribution of tracer in the brain must remain essentially stable during the period of image acquisition. In most body tissues the cellular membrane (endothelium) lining the blood capillary wall is permeable, allowing free passage of all substances up to the size of albumin between the blood and the interstitial fluid. In this way substances which are not extensively bound to high molecular weight blood components may freely enter the majority of body tissues.

This permeability results primarily from the discontinuous nature of most capillary cell wall linings; small substances may pass freely through intercellular gaps. This is not the case in the brain. Tight junctions between cells of the endothelial lining prevent the passage of substances through intracellular gaps. In addition, brain endothelial cells do not (unlike non-cerebral capillary endothelia) permit transport of substances via pinocytosis [5]. These restrictions to solute mobility between the blood stream and brain have given rise to the term blood-brain barrier (BBB) for the cerebral capillary endothelium.

While active transport and facilitated diffusion mechanisms are provided for movement of essential nutrients and metabolites across the BBB, these processes are unlikely to provide access to technetium complexes. Radiopharmaceuticals capable of employing these mechanisms must possess

the strict molecular structural features which are necessary for recognition; incorporation of technetium into molecules places constraints on the shape, size and charge distribution of the resulting complexes.

Fortunately the BBB shares with all other biological membranes the facility for simple diffusion of lipid soluble molecules [6]. This mechanism permits molecules to cross biological membranes at a net rate proportional to the concentration difference between both sides of the membrane. The net direction of diffusion is from regions of high to low concentration. Unlike carrier mediated processes, simple diffusion does not require the expenditure of energy, it is not saturable, nor is it inhibited by other substances [6]. The initial distribution of such compounds in the brain is a function of rCBF [7].

Several physical features are associated with simple diffusion. The substrate must possess high lipid solubility with little or no ionisation (charge) at physiological pH [8]. Rates of diffusion are inversely related to the molecular weight (size) of a substrate [6], and are adversely affected by high plasma protein binding [9]. Oldendorf and Kitano demonstrated that a simple lipophilic molecule, antipyrine, when labelled with iodine-131, fulfilled these requirements, and was taken up into brain following i.v. administration [10]. Regional distribution of iodoantipyrine within the brain was subsequently shown to reflect regional blood flow [11].

The BBB permits lipid-soluble substrates to cross in either direction along concentration gradients [5]. Thus, a substrate will be taken up by the brain while its concentration in the blood stream is higher than that in brain, but will diffuse from the brain when blood levels fall. Freely diffusible tracers, such as Xe-133 [12], iodoantipyrine [13] and C-11 labelled alkylalcohols [14] all display this characteristic. For these tracers of cerebral perfusion, imaging is only possible during a narrow time window either side of maximum brain uptake of the tracer.

Initial studies towards the development of a technetium cerebral perfusion agent concentrated on the discovery of complexes with the correct combination of physical properties to permit them to behave as freely diffusible tracers. However, to be useful for routine SPECT imaging (when cerebral tracer concentration must not change by more than a factor of two during image acquisition [15]) tracers with longer brain residence were required. The next section describes technetium complexes which fulfil the basic requirement to cross the BBB, and includes those complexes which, by design or luck, are also retained for sufficient time for SPECT imaging.

III COMPLEXES WHICH CROSS THE BLOOD-BRAIN BARRIER

A. Analogues of Indium Cell-labelling Agents

Several oxygen-containing ligands, such as 8-hydroxyquinoline (oxine), acetylacetone (acac) and tropolone form neutral, lipophilic complexes with indium which were shown to traverse cell membranes [16-18]. These results encouraged the investigation of analogous technetium complexes.

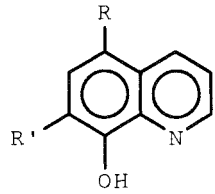
Studies involving the application of technetium complexes of oxines are summarized, in Table 1. In general, these complexes display very poor cerebral uptake following iv administration in laboratory animals, despite apparently fulfilling the basic criteria for charge and lipophilicity. High plasma protein binding appears to be the cause of the poor brain uptake of a number of these complexes [19].

Initial results obtained with technetium acac and tropolone complexes [21-23] also indicated that these complexes had poor brain uptake, apparently resulting from high blood binding. However, as shown in Table 2, recent data from studies in mice demonstrate that ^{99m}Tc-acac has high blood binding, but is extracted by the brain.

These studies confirm that low and/or weak association with circulating blood components is an essential requirement for brain uptake. Variation in

brain uptake between species may result from differences in binding to circulating blood components.

Table 1. Brain Uptake, Lipophilicity, Protein Binding, and In-Vivo Biodistribution of Tc-99m Oxine Complexes



R	R'	P	BUI(%)	pb(%)	Biodistribution of Tc-99m Oxine		
H	H	23.6	53.8	96.8		2 min	120 min
I	I	78.5	68.6	98.6	Brain	0.09	0.02
SO_3H	H	0.02	10.8	90.9	Heart	0.72	0.12
X	H	0.05	13.9	64.2	Blood	6.48	2.13
X = $(CH_3)_2CHNHCH_2CHOH$ (from Ref. 19)					Liver	31.63	22.0
					% Injected Dose/Organ in Rats (from ref. 20)		

Table 2. Biodistribution of ^{99m}Tc-acac in mice

	1 min	2 min	5 min	30 min	120 min	300 min
Brain	0.87	0.91	0.54	0.23	0.06	0.04
Heart	1.05	0.87	0.70	0.47	0.19	0.15
Blood	26.6	17.8	15.4	11.1	2.94	1.66
Liver	13.5	13.7	13.2	13.0	9.6	6.0

B. Dithiasemicarbazone (DTS)

The dithiasemicarbazone (DTS) ligand (Figure 1) forms a mixture of neutral and charged lipophilic complexes with technetium [25], only under carefully controlled conditions will the neutral lipophilic form be produced as the major component of the mixture [26]. While animal studies indicate that

lipophilic DTS complexes can cross the BBB [26], the restrictions on complex formation will preclude its widespread applicability.

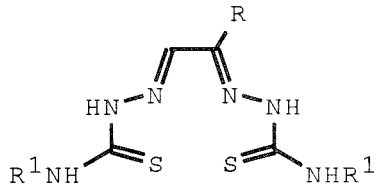


Figure 1. General Structure of DTS Derivatives

C. Diamine Dithiol (N_2S_2) Ligands

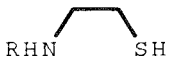
For several years, the potential of nitrogen/sulphur and nitrogen/oxygen ligand systems have been examined for the formation of neutral technetium complexes. The bidentate aminoethane thiol [27] and tridentate bis(mercaptoethyl)amine [28] both gave neutral technetium complexes, provided no charged groups were attached to the nitrogen atom (Fig. 2). The relative ease of synthesis of these ligands permitted a range of derivatives to be prepared, in which lipophilicity was varied by attachment of alkyl substituents of varying size. N-methyl bis(mercaptoethyl)amine provided a neutral, lipophilic (P=0.45) complex, which, following i.v. administration, demonstrated brain uptake in mice [28].

However, there were indications that these ligands might provide unstable technetium complexes. Challenge studies with dimercaptoethane indicated rapid ligand exchange [29]. An analogous ligand system, bis(hydroxyethyl)-ethylenediamine, also gave neutral technetium complexes which were susceptible to ligand exchange [30]. These in vitro characteristics raised doubts about the ability of these complexes to perform satisfactorily in vivo. However, the tetradentate analogue of the aminoethanethiols, the diamine

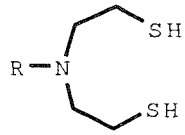
dithiol (N_2S_2) ligands provided neutral technetium complexes [29], less susceptible to ligand exchange and able to provide transient, flow-related brain uptake following i.v. administration in a monkey and in rats [31].

As the N_2S_2 ligand system has been examined by several research groups, it is reported in the literature under several different abbreviated names

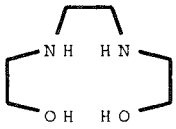
(DADT, BAT, PAT, EDAT). This has lead to some confusion over the relationship between the various N_2S_2 which have been reported. In this article, the term N_2S_2 is used to encompass all ligands based on 3,6-diaza-1,8-octanedithiol and 3,7-diaza-1,9-nonanedithiol (Fig 2).



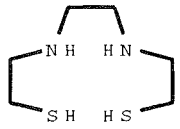
Aminoethanethiols

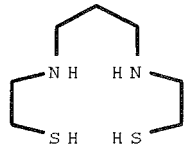


Bis(Mercaptoethyl)amines



Bis(hydroxyethyl)-ethylenediamine





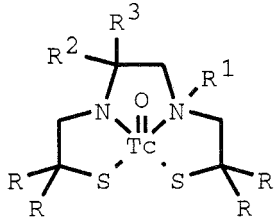
Diamine Dithiols (N_2S_2)

Figure 2. Nitrogen/Sulphur, Nitrogen/Oxygen Ligand Systems Providing Freely Diffusible Technetium Complexes

Table 3 provides a summary of N_2S_2 ligands which contain hydrocarbon substituents. Such variations alter the size and lipophilicity of the resulting

^{99m}Tc complexes, which appears to influence absolute brain uptake but has little effect on cerebral retention.

Table 3. Influence of Lipophilicity on the Brain Uptake/Retention of Tc-N_2S_2 Derivatives



SUBSTITUENTS							% ID in Brain of Rats at Times Post Injection	
Ref	R	R1	R2	R3	Log P	M Wt	2 Min.	15 Min.
31	CH_3	H	H	H	1.91	348	1.96	0.26
31	CH_3	H	CH_3	CH_3	2.32	376	2.77	0.26
31	C_2H_5	H	H	H	2.73	404	2.92	0.34
32	CH_3	H	H		2.41	455	2.3	0.3
32	C_2H_5	H	spirocyclohexyl		2.58	472	2.2	0.7

1. AMINE DERIVATIVES

Initial attempts to produce N_2S_2 derivatives which provide brain retention involved the synthesis of ligands containing amine sidechains. The selection of amine containing substituents stemmed from earlier successes with the lipophilic amines, p-iodo-N-isopropylamphetamine (IMP) and N,N,N'-trimethyl-N'-[2-hydroxy-3-methyl-5-iodobenzyl]-1,3-propane-diamine (HIPDM) as carriers of iodine-123. These compounds have cerebral uptake and retention sufficient to permit SPECT imaging [4]. Addition of aminoalkyl substituents to the carbon skeleton of N_2S_2 ligands was studied by Kung et al [33] and Kato-Azuma et al [34]. The former group placed a substituent containing one or

more amine at the 4-position of 3,6-diaza-1,8-octanedithiol, while the later placed amine substituents at the 2-, or 2- and 7-positions of this ligand. The two series of complexes showed similar characteristics:

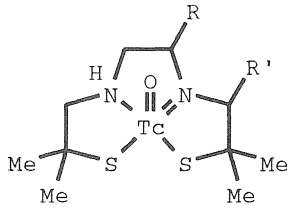
-Diamines had poor brain uptake.

-Brain uptake of monoamines appears to be related to lipophilicity.

-Monoamines provided two complexes, with the amine sidechain either syn- or anti- to the oxygen atom of the TcO core.

The stereoisomeric complexes display different lipophilicities, permitting their separation by reversed-phase HPLC [35]. As shown in Table 4, syn- and anti-geometrical isomers can display quite different levels of brain uptake in rats.

Table 4. Comparison of the Brain Uptake of N_2S_2 Stereoisomer Complexes.



BAT derivatives (ref. 35,36)
A. R=CH_2NEt_2; R'=H
B. R=CH_2-N-(N'-benzylpiperazine); R'=H

EDAC derivatives (ref 34)
C. R=H; R'=CH_2-N-(piperidine)
D. R=H; R' = CH_2-N-(4-methylpiperidine)

Compound		Brain uptake (% dose/organ) 2 m	15 m	log P
A	syn *	2.27	0.64	2.49
A	anti *	0.99	0.26	2.31
B	syn *	2.77	1.08	2.96
B	anti *	0.58	0.27	2.88
	* see Fig 3			

Compound	HPLC fraction	Brain uptake (% dose/organ) 2 m	20 m	60 m
C	#1	1.13	0.32	0.12
C	#2	0.29	0.17	0.14
D	#1	0.93	0.31	0.10
D	#2	0.38	0.24	0.11

Geometrical stereoisomerism also appears to influence the behaviour of the N-substituted N_2S_2 derivatives first reported by Burns et al [37]. While the ligand itself (due to rapid nitrogen inversion) is symmetrical, on complexation with technetium, nitrogen inversion is halted, and the side arm attached to the nitrogen atom is locked into either a syn- or anti-position relative to the technetium-oxo core (demonstrated in Fig. 3). This provides two stereoisomers, denoted A and B, which may be separated by HPLC and evaluated independently. The N-ethylpiperidinyl ligand (NEP-DADT) gives the A and B complexes in a ratio of 80:20 [37]. Examination of the Tc-99 complexes of NEP-DADT revealed the A complex to have the syn-configuration, while the B complex has the side chain anti to the oxo core [38].

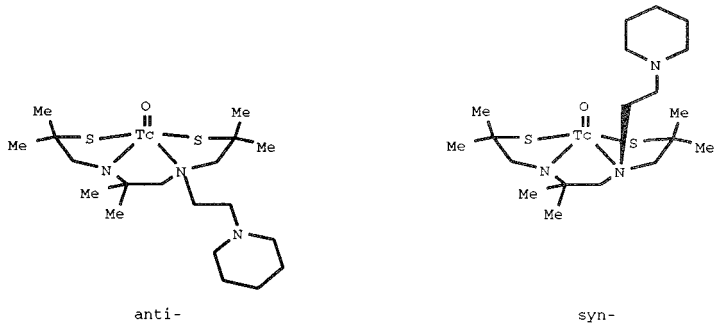


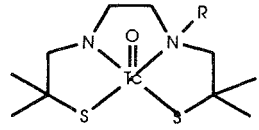
Figure 3. Syn- and Anti-Tc-99m Complexes of NEP-DADT

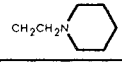
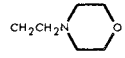
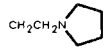
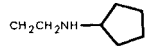
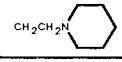
The more lipophilic A complex displays the greater the brain uptake and longer washout halftime. In primates, the brain washout rates ($t_{1/2}$) of this complex is approximately 60 minutes [37]. Similar rapid washout rates from brain were observed in clinical studies [39]. Reasonable SPECT images of cerebral perfusion can be obtained up to 15 minutes post injection of hplc-purified Tc-99m NEP-DADT, but thereafter, washout and redistribution degrade image quality. Bok reported [40] that a 4-methyl substituent on the piperidine ring of NEP-DADT gave a complex with superior brain retention than the NEP-DADT 'A' complex.

Chiotellis et al [41, 42] observed that ligands analagous to NEP-DADT (See Table 5) each provide up to four technetium complexes. For each ligand, it is the most lipophilic of the four complexes (separated by HPLC) which displayed the greatest brain uptake and retention, and it is the brain uptake results on these complexes which are shown in Table 5. Of these new derivatives the pyrrolidine analogue appears to display the best overall characteristics of brain uptake and retention.

Clearly, this area has shown some promise, but addition of an amine substituent to the N_2S_2 backbone, either to a nitrogen or carbon atom, gives rise to stereoisomer mixtures. The necessity for HPLC purification to isolate the preferred technetium complex from that mixture will exclude any amine-N_2S_2 from routine use.

Table 5. Brain Uptake of Tc-99m Complexes of N_2S_2 Derivatives With N-Linked Amine Sidechains

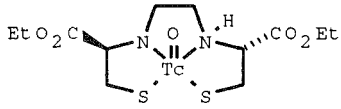


Substituents			Brain Uptake in Mice		
Ref.	R	Isomer	% ID/organ		
			5 min	15 min	30 min
38		A	2.19	1.01	0.53
		B	1.09	0.28	0.16
			% ID/g		
			2 min		15 min
41,42			7.75		1.05
41,42			11.45		4.11
41,42			8.36		3.01
41,42	$CH_2CH_2N(iPr)_2$		9.24		1.49
41,42			11.56		2.80

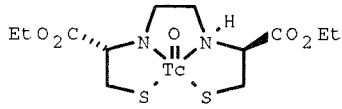
2. ECD

Ethyl cysteinate dimer (ECD) differs structurally from other N_2S_2 ligands studied previously; there are no substituents on the 1- and 8- positions of 3,6 diaza-1,8-octanedithiol, and the 2- and 7- positions are substituted, with ethoxycarbonyl groups. While the ligand was first reported in 1967 [43], its technetium chemistry has been studied only recently [44].

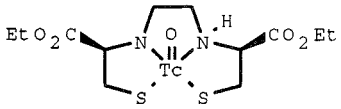
The structures of the technetium complexes of ECD are shown in Fig 4. The ligand has two chiral centres, resulting in three stereochemical forms, dependent upon whether the ligand was synthesized from the 'natural' L-aminoacid, or the 'un-natural' D-form, or a dimer composed of D and L forms, giving the meso-ligand.



Tc-99m L,L-ECD



Tc-99m D,D-ECD



Tc-99m D,L-ECD

Figure 4. Tc Complexes of ECD Stereoisomers

In Rhesus monkeys, all three complexes display reasonable brain uptake, but only the L,L-dimer complex displays good brain retention [45]. The D,D-complex behaves as a freely diffusible tracer, whereas the meso-complex displays delayed cerebral washout [46].

The high brain retention of L,L-ECD is seen only in primates and man; in lower species results from this complex show it is not retained in the brain [46]; Table 6 compares the biodistribution of ^{99m}Tc-L,L-ECD in rats and man. Results of studies which have tried to elucidate the cause of the species and stereochemical differences of ^{99m}Tc-L,L-ECD are described later in this chapter. In preliminary clinical studies, this radiopharmaceutical has shown promise for routine SPECT rCBF imaging [48-50]

Table 6. Comparison of the Biodistribution of Tc-99m L,L-ECD in Rats, Monkeys and Man

	Rats (ref 47)		Monkeys (ref 45)		Man (ref 49)	
	2 min	60 min	10 min	60 min	5 min	60 min
Brain	0.82	0.08	4.8	4.7	6.4	5.2
Blood	7.45	0.85	6.0	2.7	-	1.1
Lungs	1.30	0.30	-	-	4.6	1.4
Liver+GI tract	36.88	52.23	17.4	18.6	15.9	8.8
Kidneys+Urine	3.56	41.17	18.5	34.3	17.1	25.4

D Propyleneamine Oxime (PnAO) Ligands

While studying the tetra-aza macrocyclic ligand system cyclam, which provides a Tc(V) complex with an overall +1 charge [51], Troutner and Volkert recognised the potential for propyleneamine oxime (PnAO) [52], another N_4 ligand, to form an analogous complex in which, by deprotonation during complex formation, the overall charge would become zero.

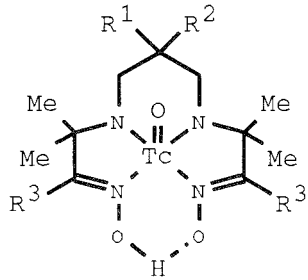
The technetium PnAO complex possesses an octanol/saline partition coefficient of 57.7, reversible binding to plasma proteins and red blood cells, and demonstrates 1.3% of injected dose taken up within the brain of rats 15 secs post i.v. injection [53]. The first pass brain extraction efficiency of PnAO was estimated to be 80% [53]; this provides an initial flow-related distribution of ^{99m}Tc-PnAO, determined by autoradiography with rats [54] and dynamic SPECT in man [55]. As was the case with the N_2S_2 ligands, several

derivatives of PnAO containing hydrocarbon substituents were prepared; these changes influenced brain uptake, but had no effect on retention.

1. AMINE DERIVATIVES

The rationale for synthesis of PnAO derivatives with amine sidechains is identical to that described for the N_2S_2 ligands; to mimic the cerebral retention of HIPDM and IMP. A range of amine-PnAO derivatives were synthesized and their technetium complexes evaluated in rats. The structures of the technetium complexes from these ligands are shown in Table 7. All these complexes failed to show brain uptake.

Table 7. Brain Uptake in Rats, log P and M. Wt. of PnAO Complexes With Amine Sidechains [ref. 56]



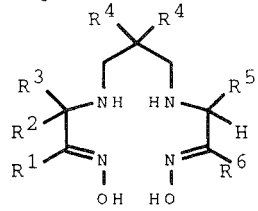
R1	R2	R3	Brain Uptake	LogP	Complex M.Wt.
N-piperidinyl	H	CH_3	0.12	-	467
N-piperidinyl	H	n-C_4H_9	0.27	-	553
N-morpholinyl	H	CH_3	0.17	0.34	469
N-morpholinyl	H	C_2H_5	0.14	-	497
2-N-morpholinyl ethyl	H	CH_3	0.20	0.5	497
2-N-ethylanilino ethyl	H	CH_3	0.03	2.75	531
2-pyridyl	H	CH_3	0.1	-	461
amino methyl	CH_3	CH_3	0.06	0.16	427
amino methyl	CH_3	n-C_4H_9	0.06	2.60	511
N-isopropylamino methyl	CH_3	CH_3	0.07	0.0	469

This result is somewhat suprising considering the brain uptake seen with the amine-N_2S_2 complexes. Possible causes, such as lipophilicity, size, plasma protein binding, pK_a, of the complexes were examined [56-58], but none of the values obtained for these parameters provides a rationale for the inability of the amine-PnAO series to cross the BBB.

2. HM-PAO

For reasons still unclear, PnAO derivatives with one less alkyl substituent at either the 2-position or the 2- and 8-positions, displayed good brain uptake in rats with reduced washout rates [58,59], as shown in Table 8. From initial studies, it was found that the ^{99m}Tc complexes of TM-PAO [60,61] and the unsymmetrical ligands PM-PAO and ETM-PAO [54] possessed excellent brain uptake and retention properties in rats. However, further work in rats [62] and baboons [63] demonstrated, of the four PAO ligands, HM-PAO had greatest promise.

Table 8. PAO Derivatives and Brain Uptake of Their Tc-99m Complexes [ref. 59]



Trivial Name	R1	R2	R3	R4	R5	R6	% ID in brain of rats 2 min	60 min
TM-PAO	CH_3	H	CH_3	H	CH_3	CH_3	1.35	1.34*
HM-PAO	CH_3	H	CH_3	CH_3	CH_3	CH_3	1.39	1.05
PM-PAO	CH_3	CH_3	CH_3	H	CH_3	CH_3	1.38	1.32
ETM-PAO	CH_3	CH_3	CH_3	H	CH_3	C_2H_5	1.49	1.35

* Sacrifice at 120 mins.

As shown in Fig. 5 HM-PAO possesses three stereoisomeric forms, d-, l- and meso. Ligand synthesis produces an equal mixture of the two diasteroisomers (d,l and meso) and the initial studies with HM-PAO made use of this mixture. When the diastereoisomers were separated and individually labelled, they displayed strikingly different biodistribution properties as shown in Table 9.

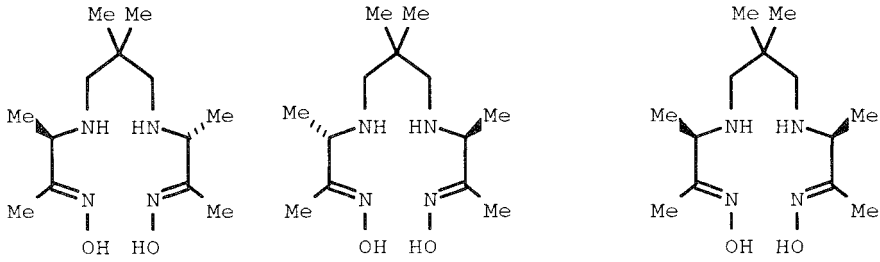


Figure 5. HM-PAO Diastereoisomers

The d,l-diasteroisomer complex displays far superior brain retention (Table 9) with minimal redistribution (Fig. 6); by comparison the meso-complex shows rapid washout with redistribution [64]. The regional distribution of ^{99m}Tc d,l HM-PAO within the brain reflects cerebral perfusion [65], a conclusion supported by an independent study [66]. What little data has been reported on possible differences between the d- and l-enatiomers of HM-PAO indicates that the biodistributions of these complexes are not identical [67].

^{99m}Tc dl-HM-PAO has been the subject of extensive clinical trials, demonstrating potential for diagnosis, study and prognosis in a variety of

neurological disease states such as stroke and cerebral ischaemia [68], dementias [69,70], brain tumours [71], epilepsy [72] and in the confirmation of brain death [73-75].

Table 9. Biodistribution of Tc-99m-HM-PAO Diastereoisomers in Rats [64]

	% injected dose per organ at times post injection					
	2 min			10 min		
	d,l	meso	mixture	d,l	meso	mixture
Brain	2.1±0.1	1.2±0.1	1.1±0.1	2.1±0.2	0.9±0.2	0.7±0.1
Blood	11.0±0.4	11.6±0.3	5.0±0.2	10.5±1.1	8.9±1.5	3.8±0.2
Lungs	3.7±0.8	2.3±0.3	1.3±0.1	3.1±0.2	2.6±1.3	1.1±0.0
Kidneys	6.5±1.1	6.3±0.4	3.1±0.2	6.3±0.2	5.3±0.3	2.5±0.4
Urine	0.4±0.4	0.2±0.1	0.2±0.1	3.2±0.3	3.7±3.2	0.7±0.0
Liver	13.9±1.9	16.5±0.1	26.4±1.7	10.2±0.4	17.2±2.6	31.2±0.2
Intestines	15.4±1.7	12.2±0.3	13.3±0.6	15.8±0.2	12.3±2.9	19.7±1.5
Brain/Blood	1.2±0.0	0.6±0.0	1.3±0.1	1.3±0.1	0.6±0.1	1.3±0.0
Brain/Muscle	4.1±0.5	2.0±0.1	1.7±0.2	3.0±0.1	1.4±0.1	1.9±0.1

	% injected dose per organ at times post injection					
	30 min			60 min		
	d,l	meso	mixture	d,l	meso	mixture
Brain	1.8±0.2	0.6±0.1	0.7±0.0	2.0±0.2	0.7±0.1	0.6±0.1
Blood	9.4±0.2	5.9±0.3	3.4±0.1	8.6±0.3	4.6±0.2	2.8±0.1
Lungs	3.1±0.1	1.5±0.4	0.8±0.1	2.8±0.3	0.8±0.0	0.7±0.1
Kidneys	6.6±0.4	4.9±0.3	2.7±0.1	6.4±1.2	4.8±0.1	2.6±0.1
Urine	9.8±0.1	16.1±1.3	3.2±0.2	14.6±1.3	19.8±0.2	7.4±0.0
Liver	10.4±0.4	18.3±1.1	28.9±2.9	8.6±1.0	17.3±0.1	25.6±0.4
Intestines	19.8±0.3	22.2±1.1	32.1±2.8	21.5±1.5	28.1±2.4	36.9±1.8
Brain/Blood	1.3±0.1	0.7±0.1	1.3±0.0	1.5±0.1	0.8±0.0	1.5±0.1
Brain/Muscle	3.8±0.7	2.2±0.3	2.4±0.2	5.7±1.4	2.8±0.6	2.9±0.0

Results are the mean ± S.D. of three animals

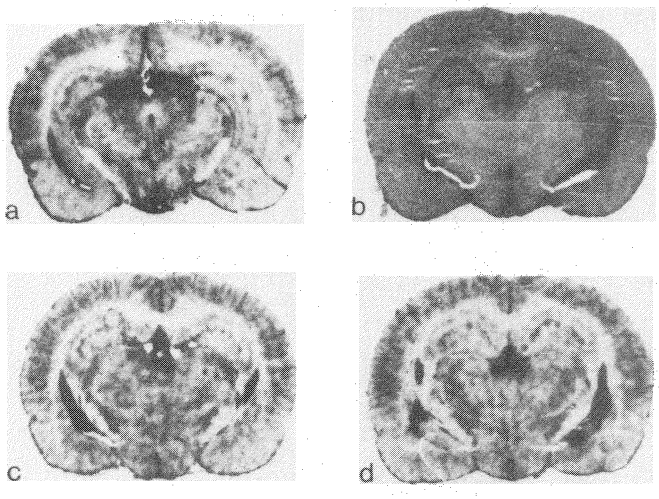


Figure 6. Rat Brain Autoradiography of Meso and d,l-HM-PAO Complexes

E. $^{99m}TcCl(DMG)_3 2MP$

The BATOs (Boronic Acid adducts of Technetium diOximes) are a novel series of Tc(III) complexes formed by the stannous reduction of $^{99m}TcO_4^-$ in the presense of a vicinal dioxime and a boronic acid at low pH/100^{0}C [76]. Unlike other transition metal oxime/boronate chelates, which have two boron caps [77], the heptacoordinate BATOs have a single boron cap, and an axial monodentate ligand (Fig. 7), with a net zero charge. BATO complexes are formed in high yield in a one step process. Two intermediates have been identified [78]; a tin-capped tris(dioxime) technetium complex first described by Deutsch et al [79], and an uncapped tris(dioxime) complex. Failure to cap

both ends of the complex has been attributed to the size of the technetium core, and seven coordinate geometry [80].

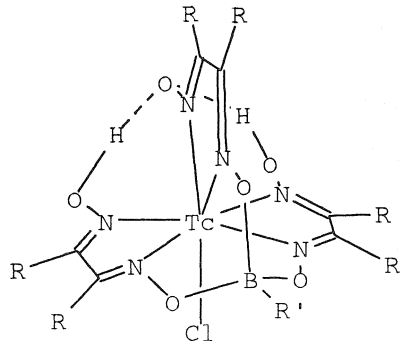


Figure 7. Structure of BATO Complexes

As described elsewhere in this volume [81], a large number of BATO complexes were made and evaluated. One of these complexes (produced from dimethylglyoxime and s-butyl boronic acid, and named as SQ 32,097 or $^{99m}TcCl(DMG)_32MP$), was selected as the most promising for cerebral perfusion imaging [82]. $^{99m}TcCl(DMG)_32MP$ has high cerebral extraction in rats, and in baboons, 2.75% of injected dose is in the brain 5 minutes after iv injection [83]. In rat and cynomologus monkey, autoradiography reveals that this complex provides excellent descrimination of grey and white regions of the brain (Fig 8.). The cerebral washout half time in baboons is 100 minutes, sufficiently slow to allow gamma camera SPECT imaging [83]. In clinical studies, $^{99m}TcCl(DMG)_32MP$ is demonstrating potential for routine use as a cerebral perfusion imaging tracer.

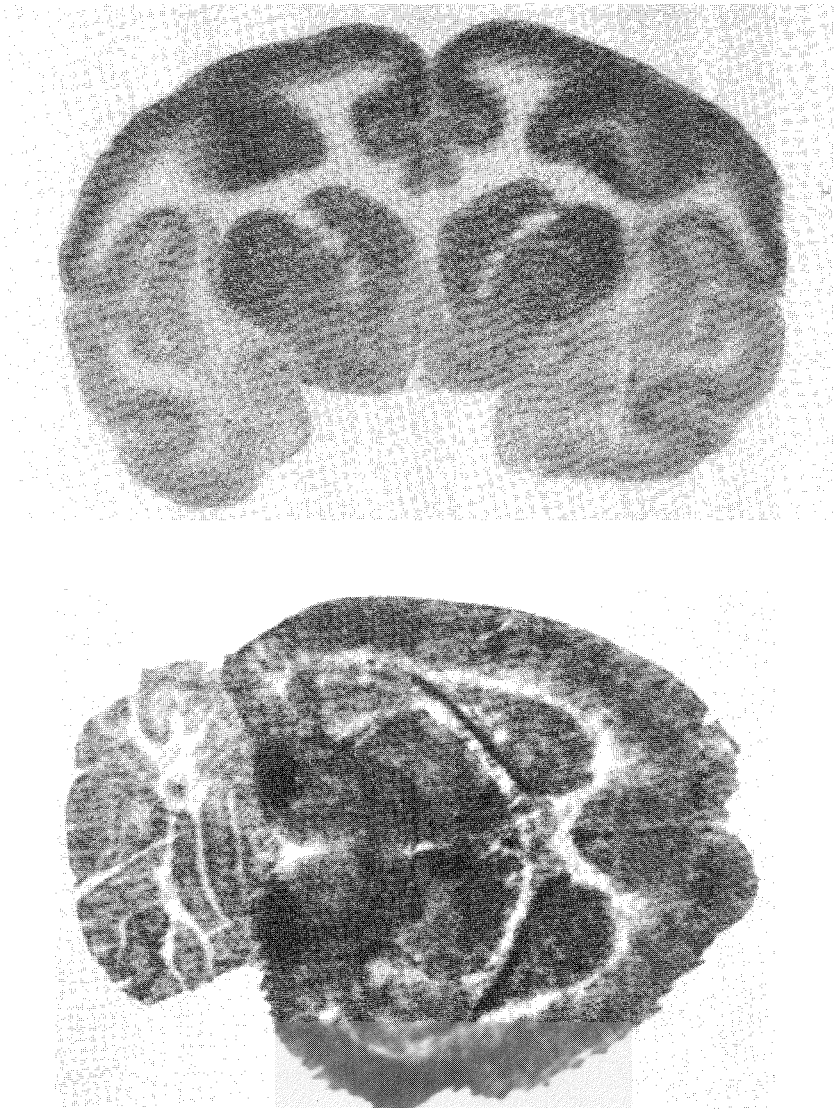
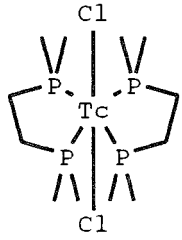


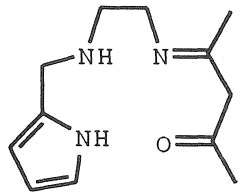
Figure 8. Monkey and Rat Brain Autoradiographs of $^{99m}TcCl(DMG)_32MP$.

F. Other Ligands

Although the complexes ^{99m}Tc(Cl)DMG-2MP, ^{99m}Tc-ECD, and ^{99m}Tc-d,l-HM-PAO are currently either in clinical trials, or commercially available for cerebral perfusion imaging, studies with the aim of developing new ligands for this clinical application are still in progress. This section describes some of those new complexes. The structures of these complexes or ligands are shown in Figure 9.



Neutral Tc(II) complexes



MRP20

Figure 9. Structures of New Tc-99m rCBF Tracers

Reduction of the cationic Tc(III) complexes of dimethylphosphinoethane (DMPE) and o-phenylene-bis(dimethylarsine) (DIARS) [84], with sodium borohydride gave the corresponding neutral Tc(II) complexes [85]. The neutral complex from DIARS displays modest brain uptake in rats following intravenous administration. During tracer clearance from the blood stream, a constant brain/blood ratio is maintained, suggesting that this is a freely diffusible tracer. The more lipophilic Tc(II) complexes from diphenylphosphinoethane.

(DPPE) [85] and diethylphosphinoethane (DEPE) [86] show no brain uptake. High plasma protein binding is given as the reason why these compounds fail to penetrate the BBB.

Thornback et al have described a new tetradentate ligand, MRP20, which forms a neutral, lipophilic complex with ^{99m}Tc [87]. Initial results with this complex suggest that it has several features in common with ^{99m}Tc-d,l-HM-PAO; ^{99m}Tc-MRP20 shows good brain uptake and retention in rats, but the radiopharmaceutical purity deteriorates on standing. Also demonstrating promise in preliminary animal studies are synthetic ionophores labelled with ^{99m}Tc [88].

IV. FACTORS INFLUENCING BRAIN UPTAKE AND RETENTION

A Blood-Brain Barrier Penetration

1. LIPOPHILICITY

Dischino et al [14] found that lipophilicity had a profound influence on the brain extraction efficiency of C-11 labelled alcohols and ethers in baboons. Percentage brain extraction rose linearly in the range of log P values -1 to +1, remained constant over the range log P 1.0-3.0, then declined in a linear fashion as lipophilicity was increased further still. This parabolic profile is similar to that found by Hansch and co-workers [89] for biological activity of related CNS drugs and log P; pharmacological activity was equated with delivery to the active site, and this study indicated that a log P value of 2 is optimum for passive diffusion across the BBB.

A similar trend was observed in a homologous series of alkyl PnAO derivatives [58]. The lipophilicity of these complexes, as measured by an HPLC method [58], increases linearly with alkyl chain length (Fig. 10) as might be predicted from Hansch fragment constants [90]. The first four members of this series, with log P values in the range 1 to 4 show similar brain uptake in rats, but higher homologues, with log P values greater than 4, show no brain uptake (Fig 11). A sequential fall of brain extraction efficiency with increasing chain length was observed for the lower four members of this series [91].

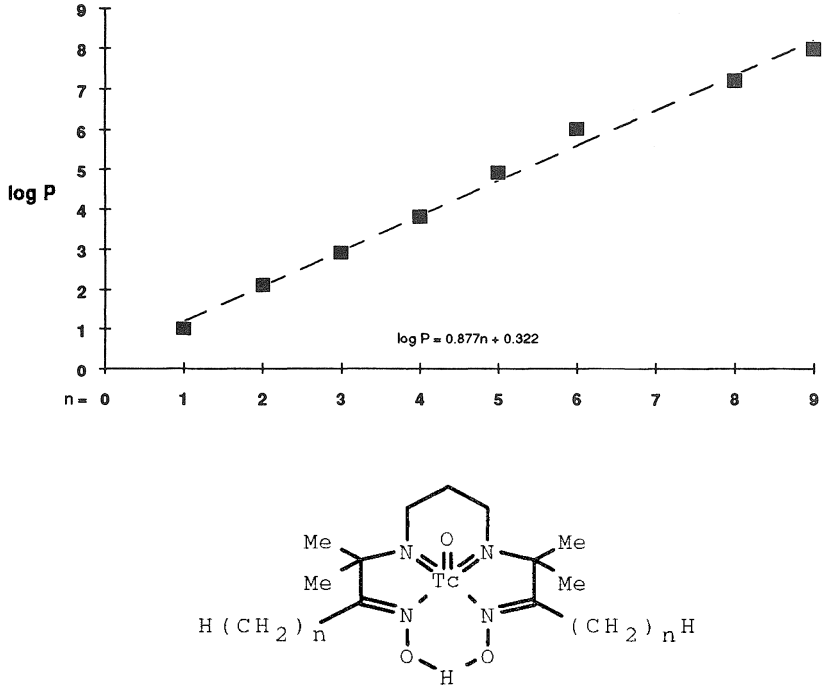


Figure 10. Graph of Log P (Determined by HPLC) Vs Alkyl Chain Length

It is well accepted that observed improvements in cerebral uptake achieved by increasing lipophilicity result from easier penetration of the lipid bilayer of the BBB. This is valid only when compounds are not highly bound to circulating blood components, and are free to interact with the BBB.

Hansch and coworkers have observed a linear relationship between log P and albumin binding [92]. Similarly, in the evaluation of plasma protein binding of a large number of Tc-99m PnAO complexes (Fig 12), a rapid rise in plasma protein binding is observed on increasing lipophilicity (log P) in the

range of 0.5 to 3.0 [58]. These data are used to account for the decrease in brain uptake of the higher PnAO homologues, shown in Fig 11.

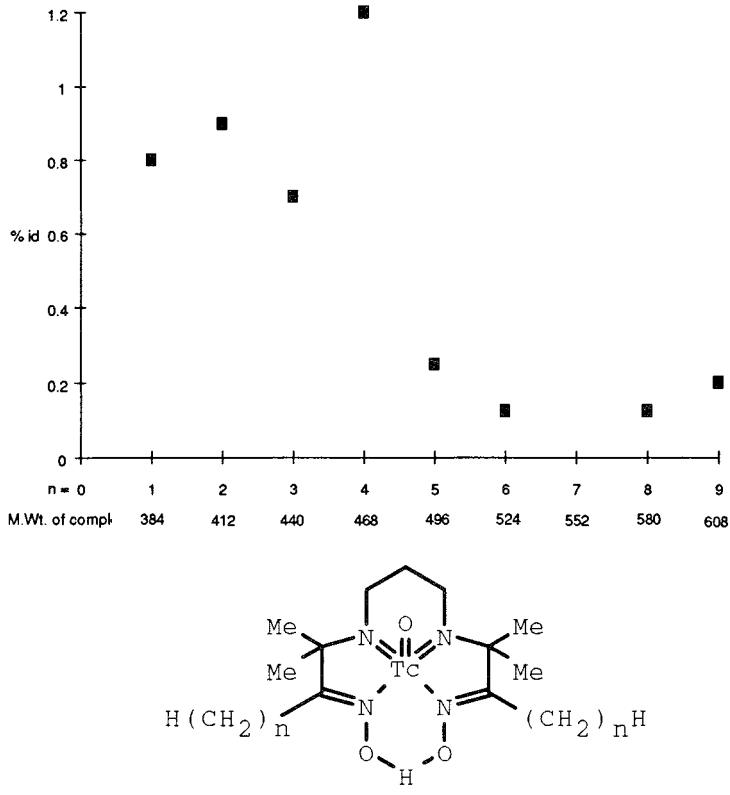


Figure 11. Graph of Brain Uptake (% ID/Whole Organ) in Rats 2 Minutes pi Vs Alkyl Chain Length [Ref. 58]

However, it is often assumed that high lipophilicity inevitably results in poor cerebral extraction due to binding to circulating blood components [93]. Results with the N_2S_2 and BATO series (Table 3 and Fig 13, respectively) would indicate that this generalisation is incorrect; highly lipophilic complexes

in both series show good brain uptake. The lipophilic BATO complexes appear to have low plasma protein binding [94]. Plasma protein binding, and (more importantly) the rate at which the protein-substrate complex is able to dissociate, are not solely reliant on lipophilicity.

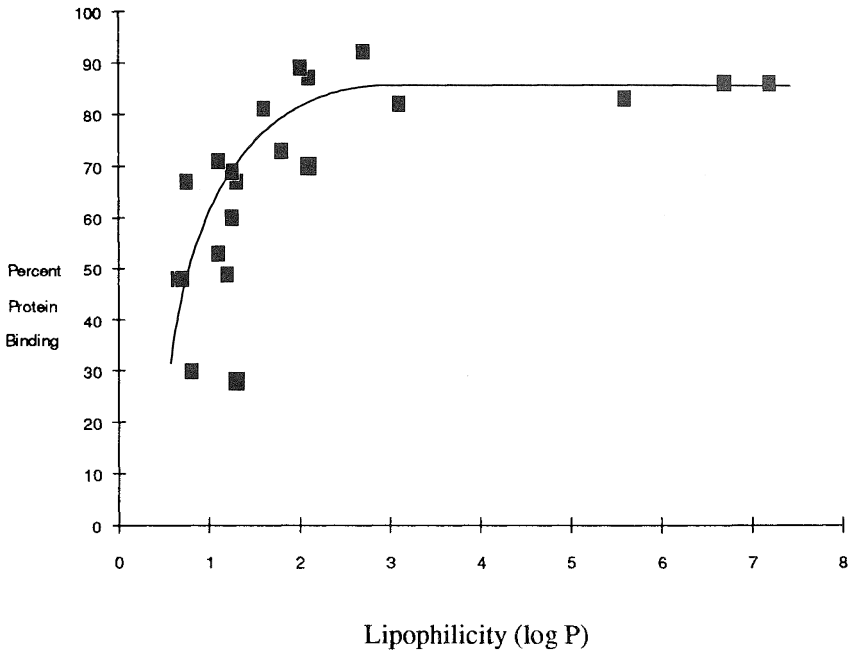
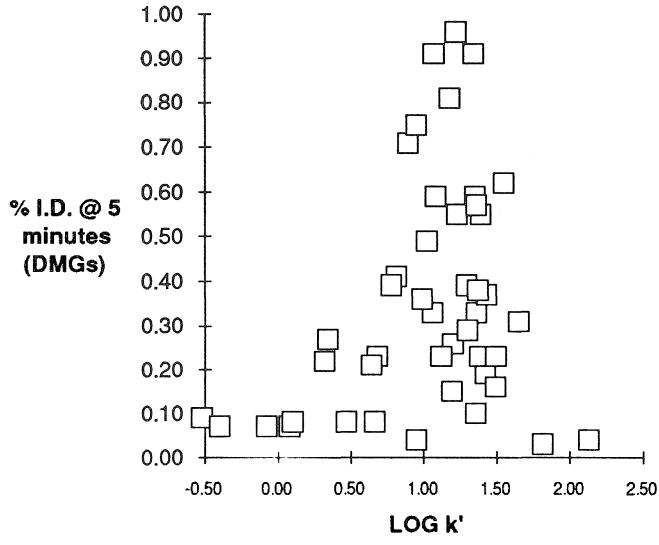


Figure 12. Graph of Plasma Protein Binding of PnAO Complexes Vs Lipophilicity

Results with the BATO series (Fig. 13) also serve to demonstrate that brain uptake is not dependent upon lipophilicity alone. While a parabolic relationship between brain uptake and lipophilicity can just be seen, the scatter of points clearly indicates that other factors influence brain uptake.



(The HPLC capacity factor (k') is linearly related to lipophilicity)

Figure 13. Graph of Brain Uptake of BATOs In Rats Versus Complex HPLC Retention (Log K') [Ref. 94]

2. SIZE AND CHARGE

While the influence of lipophilicity on drug membrane diffusion has been studied extensively, relatively little is known about other possible factors, such as size, shape and charge [94]. There have been no studies of these factors in radiopharmaceutical sciences.

Size, or molecular volume, has been shown to influence membrane transport [96]. As molecular volume (V_m) is related to molecular weight (m.wt.) by the relationship:

$$V_m = m.wt./d$$

where d is density, m.wt. has been used as a first approximation to volume in membrane diffusion studies. It must be remembered, however, that studies attempting to correlate membrane diffusion with m.wt. have an intrinsic error; the densities of all compounds studied will not be the same.

Levin [97] examined the capillary permeability coefficient of 27 compounds, relating his results to m.wt. and lipophilicity. For compounds with m.wt. up to 400 daltons he found good agreement between lipophilicity and capillary permeability, but above 400 daltons, regardless of lipophilicity, BBB permeability was reduced. Levin proposed an upper molecular weight limit for BBB passage of 657 daltons [97]. These data encouraged the search for technetium cerebral perfusion tracers be limited to complexes with a m.wt. below 600 Daltons. However, several compounds heavier than this arbitary limit have shown reasonable brain uptake, indicating that weight (or volume) is only one factor of several influencing brain uptake.

Charged molecules, such as the small hydrophilic cations Rb^+ and Tl^+, as well as the lipophilic technetium isonitrile cations, are unable to cross the BBB. These, and many other examples, give rise to the belief that neutrality is an essential requirement for BBB transit. While the case is clear for atoms and molecules which are wholly ionised at physiological pH, the influence of charge on membrane permeability of ionisable compounds is less certain.

The proportion of an ionisable molecule which is charged in solution is governed by the difference in pK_a of the molecule and solution pH [98]. For a base:

$$pK_a - pH = \log ([\text{ionised form}]/[\text{neutral form}])$$

It was proposed that molecules must possess little or no charge if they are to cross the BBB [8]. The amphetamines demonstrate that this assumption is incorrect. The secondary amine in IMP has an estimated pK_a of 11.2 [57]. From the above equation, it can be determined that IMP will be >99% ionised

in blood. Despite the low proportion of neutral IMP in the blood stream, this compound shows high accumulation in brain. The reasons for this are still unclear. The very rapid interconversion between neutral and charged forms is undoubtedly one factor; it provides a constant (albeit small) flux of neutral molecules to the BBB. However, it should not be overlooked that the surfaces of all biological membranes, including the BBB, are charged; this may allow the charged form of ionisable molecules to be 'held' (by electrostatic attraction) momentarily at the membrane surface, increasing the probability that the molecule will become neutral while adjacent to the membrane.

Clearly, studies of the interaction of molecules at the membrane surface will provide new insights to membrane permeability, and delivery of drugs and radiopharmaceuticals to target sites.

3. HYDROGEN BONDING

In 1967, Stein [99] proposed that the permeability constant of a lipid soluble compound penetrating a cell membrane by passive diffusion is inversely related to the number and strength of hydrogen bonds formed in aqueous solution. To enter the lipid membrane, Stein suggested that all hydrogen bonds formed between a substrate and water molecules must be broken. This factor will have its main impact in processes where the substrate has a time limited interaction with a biological membrane, for example, the blood-brain interface.

The BBB permeability of compounds in relation to hydrogen bonding has been examined, using either Stein's assignment method [99] for estimating the number of hydrogen bonds [100], or the difference between partition coefficents determined in octanol/water and alkane/water systems as a measure of substrate intermolcular hydrogen bonding [101]. It was found that BBB permeability is inversely related to hydrogen bonding.

Although there are no reported studies on the relationship between hydrogen bonding and brain uptake in technetium radiopharmaceuticals, an attempt is made here, using reported data, to demonstrate that hydrogen bonding effects may have some influence on the brain uptake of ^{99m}Tc complexes.

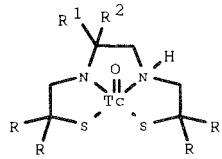
As shown in Table 10, a N_2S_2 complex with a free hydroxyl substituent possesses a partition coefficent value which should permit BBB transport, but demonstrates substantially less brain uptake compared with a primary amine analogue [32]. The primary amine N_2S_2 complex displays lower brain uptake than similar complexes with a tertiary amine substituent [33]. As strength of hydrogen bonding generally follows the series $OH > NH_2 > NR_3$, then brain uptake in this series appears to be inversly related to hydrogen bond strength. Attachment of two tertiary amine substiuents, equivalent to the HIPDM sidechain, to the N_2S_2 ligand prevents brain uptake [35]. While this result appears to support the trend of reduced brain uptake due to attachment of hydrogen bonding groups, another diamine substituent, a derivatized piperazinyl group, does not reduce brain uptake of the corresponding N_2S_2 technetium complex in rats [36]. Perhaps this complex forms intramolecular hydrogen bonds, so reducing its intermolecular hydrogen bonding.

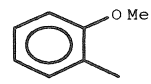
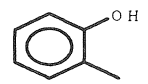
In the two phenyl N_2S_2 derivatives shown in Table 10, lipophilicity of their technetium complexes differs by an amount which might be anticipated from the difference in structure - a CH_2 group. Both complexes display lipophilicity values which should enable them to cross the BBB, but the complex with the hydroxyl group displays only slight brain uptake. The relative hydrogen bonding of the hydroxyl and ether functional groups may account for this difference. For comparison, data are provided in Table 10 on a simple alkyl N_2S_2 complex, the spirocyclohexyl N_2S_2. This displays similar lipophilicity and brain uptake to the methoxyphenyl N_2S_2 complex, providing

some confirmation of Stein's assignment [99] of a zero contribution to overall solvent hydrogen bonding by the ether functional group.

Clearly, at this stage, it is only possible to speculate on hydrogen bond effects in BBB permeability, and further studies will be required to confirm the trends described here.

Table 10. Influence of Hydrogen Bonding on the Brain Uptake in a Series of Tc-99m N_2S_2 Derivatives



R	R^1	R^2	Brain uptake*	log P
CH_3	H	CH_2OH	0.4	1.80
CH_3	H	CH_2NH_2	1.3	1.67
CH_3	H		2.3	2.41
CH_3	H		0.4	2.29
C_2H_5	Spirocyclohexyl		2.2	2.58

* % ID in Brain of Rats. 2 min post injection

B. Retention Mechanisms

Fig. 14a demonstrates the characteristics of a freely diffusible tracer. Following i.v. administration the blood:brain concentration gradient favours a net inflow of tracer to the brain compartment. Removal of tracer from the

blood stream, especially by the excretory organs, can rapidly reduce the blood concentration of tracer. Once the concentration in the blood stream is less than that of brain, there is a net outflow of tracer from the brain.

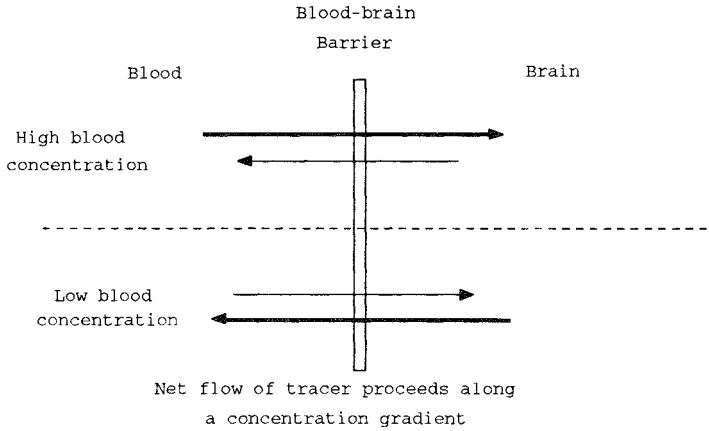


Figure 14. Diffusion Across the BBB-Freely Diffusible Tracer

As the net rate of transfer in either direction depends upon the difference in concentration between the adjoining body compartments an obvious possible strategy for achievement of delayed washout might be to aim for freely diffusible tracers with reduced blood clearance rates. This can easily be ruled out. The net rate of transfer of a freely diffusible tracer disguises the rapid diffusion of tracer molecules in both directions across the BBB. While regional cerebral uptake of a tracer in its first pass following administration will reflect blood flow, a steady state achieved through an equilibrium of tracer molecules between blood and brain will be dependent upon other physiological factors [6]. This will result in a redistribution of tracer molecules with loss of high/low flow contrast.

Hence, to maintain the flow-dependent distribution of tracer resulting from the first pass uptake, the ability of the tracer to cross the BBB must be curtailed immediately following its cerebral uptake. In Fig. 15 two possible solutions are shown.

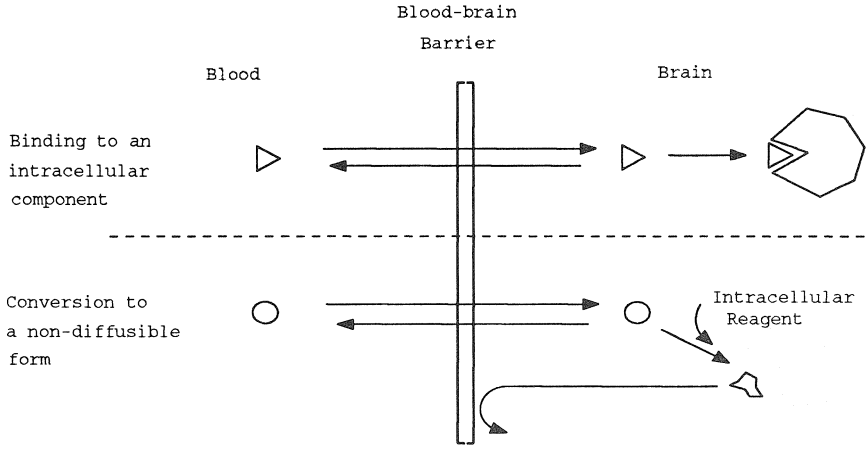


Figure 15. Diffusion Across the BBB-Inhibition of Washout

If the molecule has a high affinity for some intracellular component, then binding to this component will prevent the tracer's return to the blood stream. Alternatively, if the molecule can, in some way be chemically modified such that one or more of the essential physical requirements for BBB diffusion is lost, then the tracer will be trapped.

Any modification to the cores to introduce features which might aid brain trapping must not adversely influence the ability of these ligands to carry technetium across the BBB. To preserve electrical neutrality on complexation, modifications to PnAO and the BATOs are limited to the carbon skeleton only, and to the carbon skeleton and/or one nitrogen atom in the case of N_2S_2 ligands. A large number of N_2S_2 and PnAO derivatives have been prepared

with the purpose of improving cerebral retention with one or both of these mechanisms.

1. AMINE DERIVATIVES

The mechanism by which amine derivatives are retained is the subject of some controversy; two mechanisms have been proposed. It is well known in the field of pharmacokinetics that the distribution of an ionisable substance will be influenced by a difference in pH of two compartments separated by a semipermeable membrane [102]. Kung and Blau suggested that this phenomenon, which they termed 'pH shift' [103] might be applied to brain entrapment of radioactive tracers, as the intracellular pH within the brain is lower (pH 7.1) than that in blood (pH 7.4).

The principle of the pH shift is displayed in Fig. 16 [103]. A weak base, such as an amine with pka close to 7.4 will exist in the blood stream in equilibrium between its protonated and non-protonated forms. If the neutral form can cross the BBB, then, in this higher pH environment, a new equilibrium is established in which a far greate proportion of the amine is in its protonated, non-diffusible form. This disturbs the normal partitioning of a lipid substance between blood and brain compartments and can increase significantly the amount of substrate taken up by the brain.

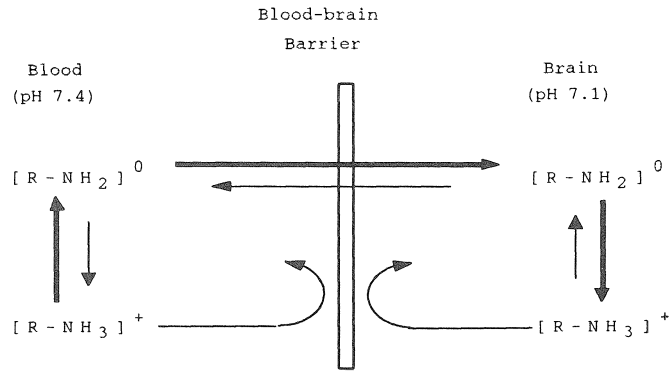


Figure 16. The pH Shift Mechanism

However, as the 'pH shift' mechanism only serves to create a new equilibrium which favours the brain compartment, it cannot be wholly responsible for brain retention [104]. The substrate will not be trapped; it remains free to diffuse back across the BBB and so can display cerebral washout, and redistribution within the brain. These considerations have led others to propose that additional mechanisms such as metabolism or non specific receptor binding are responsible for the observed properties of IMP and HIPDM [105].

2. PROPOSED IN VIVO CONVERSION MECHANISMS

Intracerebral conversion from a diffusible to a non-diffusible form is the suggested (for ^{99m}Tc d,l-HM-PAO and ^{99m}Tc ECD) or investigated (for $^{99m}TcCl(DMG)_3$2MP) mechanism of entrapment for the principal technetium complexes in clinical use. However, no conclusive evidence exists to support a conversion theory for any one of these complexes.

a. $^{99m}TcCl(DMG)_3$2MP

The axial chloride ligand of $^{99m}TcCl(DMG)_3$2MP is bound loosely to technetium [106], and may be replaced by other monodentate anions [80,107]. The chloro to hydroxy exchange under 'physiological' conditions (pH 7.4, 37^{o}C) has a $t_{1/2}$ of 21 minutes. While this rate would be too slow to account for trapping, the possibility that a more rapid rate may occur in vivo was investigated [108]. Studies using rat brain homogentate and ex vivo HPLC analysis of radioactive components in rat brain indicated that loss of free $^{99m}TcCl(DMG)_3$2MP was rapid. However, most of this loss resulted from binding to cell fragments, with low formation of $^{99m}TcOH(DMG)_3$2MP. In addition, it was shown that $^{99m}TcOH(DMG)_3$2MP can cross the intact BBB

[108]. Therefore, in vivo conversion to a less lipophilic form does not appear to contribute to the cerebral entrapment of $^{99m}TcCl(DMG)_32MP$.

b. ^{99m}Tc d,l-HM-PAO

One feature of the amine oxime ligand system recognised at an early stage of development was an apparent instability of certain lipophilic technetium complexes, for example, Tc-99m EnAO [109]. In these complexes, conversion from the lipophilic technetium complex to a less lipophilic species (not hydrolysed technetium or pertechnetate) was noted. In the series PnAO, meso-HM-PAO and d,l-HM-PAO, the relative rates for primary to secondary complex conversion are 1, 4, 30, respectively [110]. These conversation rates display an inverse relationship with brain retention; percentage of injected dose in human brain at 20 minutes post i.v. administration for PnAO, meso-HM-PAO and d,l-HM-PAO are <0.1, 1.6 and 4.9% respectively [111]. By administering secondary complex (formed by standing the reconstituted kit for 160 minutes [112]) or isolating the secondary complex from d,l-HM-PAO by HPLC (Fig. 17) [59,113], it was demonstrated in rats that this complex is unable to cross the BBB.

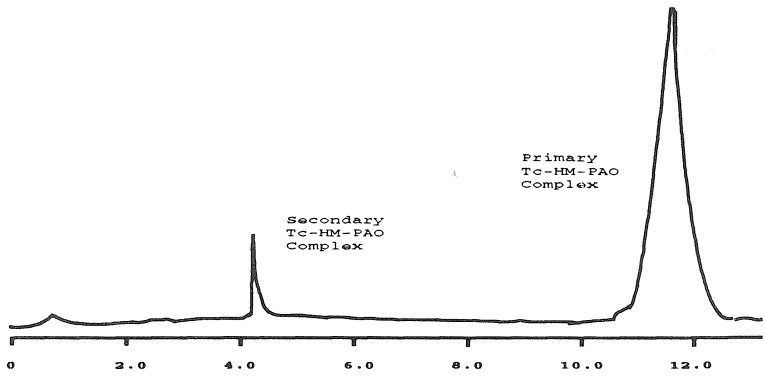


Figure 17. HPLC Separation of Tc-99m d,l-HM-PAO Into Primary and Secondary Complexes

While these results provide further support to the conversion trapping theory, the rate of conversion in saline, if duplicated in vivo, would clearly be too slow to prevent significant washout and redistribution of tracer from the brain. While the relative instability of PAO complexes in saline might reflect in vivo instability, the conversion process in vivo must be accelerated if the observed biodistribution results are to be explained by the conversion phenomenon. By analysing time activity curves of ^{99m}Tc d,l-HM-PAO in the human brain, following intracarotid injection, Lassen et al [114] have estimated the in vivo conversion rate (K_R) to be 0.85 min-1.

A mathematical model was proposed by Neirinckx [111] which provides further support to the theory that tissue trapping of ^{99m}Tc d,l-HM-PAO relies principally on the conversion process. The model assumes that the tracer is freely diffusible, the conversion product is non-diffusible, and the conversion rate is uniform throughout the body. By applying the conversion rate (K_R) determined by Lassen et al [114], excellent agreement was obtained between the observed distribution of the tracer in man, and that estimated using the mathematical model. These results are shown in Table 11.

Table 11. Percentage Uptake in Man Calculated Using a Mathematical Model and the Optimum Conversion Rate, and the Rate Determined for Tc-99m-dl-HM-PAO

Organ	$K_r = 1.05$ min^{-1} (optimum)	$K_r = 0.85$ min^{-1} (Tc-99m dl-HM-PAO)	Observed in man Tc-99m dl-HM-PAO
Brain	5.6	5.3	4.9 ± 1.3
Liver	11	11	12.0 ± 4.4
Lung	11	9.0	7.4 ± 4.9

The rate of conversion of primary to secondary d,l-HM-PAO technetium complex is influenced by reductants; the conversion rate being dependant upon the nature and concentration of reductant [115]. This observation prompted Neirinckx et al to investigate the possibility that in-vivo conversion might

result from interaction of ^{99m}Tc d,l-HM-PAO with glutathione, a reductant widely distributed in living tissue [116,117]. At levels of glutathione found typically in animal and human tissues, in vitro studies have shown that the conversion rate was raised to the same order as the in vivo conversion rate predicted by Lassen et al [114], described above. The relative rates of reaction of ^{99m}Tc meso-HM-PAO ^{99m}Tc d,l-HM-PAO with glutathione are inversely related to trapping ability [118]. Hence, glutathione may be implicated in the trapping process, although evidence has been presented which casts doubt on this proposal; there is no difference in the brain uptake of ^{99m}Tc d,l-HM-PAO in normal rats, and rats with depleted glutathione [119]; ^{99m}Tc meso-HM-PAO tissue retention appears to bear no relationship to glutathione levels [120], as should be the case if glutathione is the sole conversion reagent.

As the conversion process has a finite rate, the washout of tracer from the brain over the first ten minutes post injection probably results from back diffusion of the lipophilic primary complex, with loss of grey/white contrast. Microsphere studies in dogs [121] and rabbits [122] have shown that ^{99m}Tc d,l-HM-PAO distribution at the steady state shows good agreement with flow in low flow regions, but as the flow rate increases (as in the grey regions), the cerebral concentration of technetium does not increase as rapidly as do labelled microspheres [121]. Typical grey/white ratios of tracer in the brain of dogs was 2.1 for Tc-99m d,l-HM-PAO, compared with 3.5 with microspheres [121].

While the conversion process can be demonstrated in vitro by chromatography, the structure of the secondary complex remains elusive [115]. Initial proposals that secondary complex might result from oxime isomerism [58] were disproved [123]. The secondary complex appears not to be a single species, but a mixture of complexes [115]. In vitro studies with various reductants suggest that the rate of secondary complex formation is dependent upon the nature and concentration of reductant, and, with any given reductant, the relative proportion of the components which make up secondary complex

changes with time [115]. The ^{99m}Tc d,l-HM-PAO lipophilic complex is more stable at a pH of 7, two pH units lower than optimum for complex formation [124]. However, ^{99m}Tc d,l-HM-PAO is generally unstable to buffers, so practical implementation of a pH change method in kit form, to stabilise the lipophilic complex, might not be achieved. Radiolytic degradation can be inhibited by addition of gentisic acid after complex formation [124], or addition of iodide to pertechnetate prior to reconstitution [125].

A close analog to d,l-HM-PAO, termed d,l-CB-PAO (Fig. 18), appears to provide a complex with greater in vitro stability [126]. Stability is achieved by lowering kit pH to 7 after complex formation; unlike ^{99m}Tc d,l-HM-PAO, ^{99m}Tc d,l-CB-PAO appears to be stable in phosphate buffer. However, initial clinical results suggest that this new derivative is inferior to ^{99m}Tc d,l-HM-PAO, with lower brain uptake, and greater redistribution, resulting in poor grey/white ratios [127].

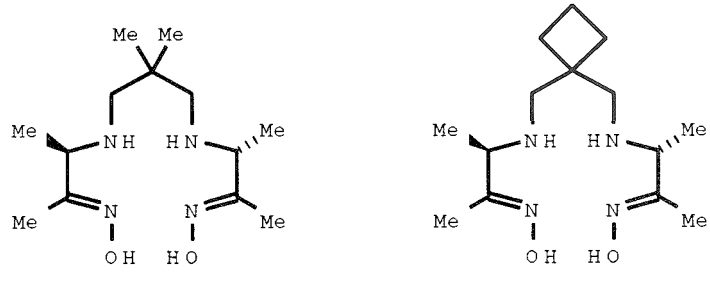


Figure 18. Structures of Tc-d,l-HM-PAO and Tc-d,l-CB-PAO

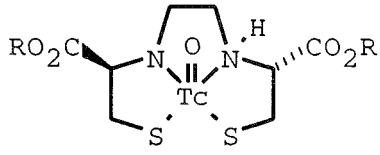
c. ^{99m}Tc L,L-ECD

The mechanism of retention of ^{99m}Tc L,L-ECD has been attributed to intracerebral de-esterification of the complex to yield either the charged, hydrophilic mono- or diacid derivative [128,129]. However, a number of

esterases [130] have been examined, and none have demonstrated the ability to de-esterify ^{99m}Tc L,L-ECD [128].

Studies with rat and baboon tissue homogenates have shown that ^{99m}Tc L,L-ECD is rapidly converted to monoacid in brain, lungs, kidneys, liver, and blood, and in kidneys, liver, and blood, the monoacid is also converted to the diacid [131]. Although the rate of conversion is ten times faster in baboon brain homogentate than rat brain homogentate, in vivo, conversion to monoester in rat brain is fast; at 5 minutes post injection, 80% of the radiotracer is in the form of monoester [131]. Therefore, it appears unlikely that the species difference in rate of conversion can account for the species difference in retention of ^{99m}Tc L,L-ECD. Furthermore, it was shown that the ^{99m}Tc diacid complex derived from L,L-ECD is able to cross the BBB [47] (Table 12), demonstrating that, in the rat, one hydrophilic metabolite of ^{99m}Tc L,L-ECD is able to cross the intact BBB.

Table 12. Biodistrubution of the Diacid Analogue of Tc-99m L,L (R,R) ECD in Rats (47)



(ECD, R = Et;Diacid, R = H)

% id/organ in rats at times following iv administration		
	2 min	60 min
Brain	0.13	0.05
Blood	44.48	13.13
Lungs	2.77	0.95
Liver+GI tract	7.21	13.38
Kidneys+Urine	7.21	46.57

As the rate of in vivo hydrolysis of ^{99m}Tc D,D-ECD and ^{99m}Tc D,L-ECD is appreciably slower than that for ^{99m}Tc L,L-ECD [128], hydrolysis rate may account for the poor cerebral retention properties of the D,D- and

D,L- isomers, but does not account for species differences. Verbruggen et al have proposed that species difference in cerebral entrapment may result from species dependent rates of cerebral elimination of the metabolite, ^{99m}Tc L,L-ECD monoacid, by a facilitated diffusion mechanism [131]. Evidence for this process is provided by the observation that, in rats, cerebral retention of ^{99m}Tc L,L-ECD is improved by the administration of probenecid [132], presumably by saturating the carboxylate transport system. A mechanism for facilitated diffusion of carboxylic acids across the rat BBB is known [133]; therefore, the proposal that differences in the rate of removal of the monoacid metabolite accounts for the species dissimilarity in cerebral retention of ^{99m}Tc L,L-ECD provides a reasonable explanation for observed species differences.

V CONCLUSIONS

Of the other areas of research directed towards a technetium rCBF agent, the greatest number of studies appear to have been devoted to amino-N_2S_2 derivatives. While the results obtained from this research have contributed to an increased awareness of the influence of physical factors, such as lipophilicity, pK_A, and stereochemistry, to radiopharmaceutical biodistribution [134], it now appears unlikely that a tracer for routine rCBF imaging will emerge from this series. Cerebral washout and redistribution rates remain greater than ideal, while the necessity for hplc purification of the ^{99m}Tc complexes would preclude routine use.

Currently, the most promising N_2S_2 derivative is L,L-ECD. While confirmation is required of its suitability for cerebral perfusion imaging in a range of perfusion disorders, initial results suggest that SPECT studies with this agent do provide flow-related images in normal volunteers and stroke patients.

^{99m}Tc d,l-HM-PAO was the first of the technetium cerebral perfusion tracers to become commercially available (in 1985). A large number of

publications now exist which demonstrate the utility of the compound, but a short post-reconstitution shelf life (30 minutes) and high backgound levels in blood and facial tissue make the agent less than ideal.

A third compound, the BATO $^{99m}TcCl(DMG)_32MP$, should also be commercially available in the future. This compound permits SPECT perfusion imaging only if tomography is completed within 30 minutes of administration, because of rapid cerebral clearance. However, the agent is stable for several hours following preparation, and the compound's cerebral clearance may prove to be an advantage in cases where repeat studies are necessary.

It is inevitable that clinical comparison of the three complexes, Tc-99m d,l-HM-PAO, ECD, and $^{99m}TcCl(DMG)_32MP$ will appear in future publications. Perhaps the most important factor in that comparison will be the relative ability of these tracers to provide a reliable 'map' of cerebral perfusion in a wide variety of disease states. This factor will be governed by uptake and trapping mechanisms. As all three tracers are neutral and lipophilic, it is assumed that they enter the brain as a result of passive diffusion. Given that these tracers have brain extraction efficiencies of the same order at normal flow levels, each should provide a similar initial flow-dominated cerebral distribution pattern. However, that initial distribution is altered by tracer washout, with cerebral washout in man following the order ^{99m}Tc d,l-HM-PAO > ^{99m}Tc L,L-ECD > $^{99m}TcCl(DMG)_32MP$. This relationship could well define the range of applications for which each agent is preferred.

As each tracer is apparently trapped by a different mechanism, there is the possibility that they may not provide exactly the same image in all disease states. Such alterations to the flow-dominated image could provide data on the underlying biochemical changes, and, in so doing, may provide the clinician with more information than provided by a 'pure' perfusion image.

Clinical studies have shown that ^{99m}Tc d,l-HM-PAO, ^{99m}Tc L,L-ECD and ^{99m}TcCl(DMG)$_3$2MP can provide 'maps' of cerebral perfusion in a wide range of disease states. With these compounds, the objective set by Oldendorf in 1978 have been achieved. To expand the application of ^{99m}Tc brain imaging, research can now concentrate on tracers which allow the distribution of the tracer to be dominated by the trapping process, rather than flow, leading to a new generation of brain agents, ^{99m}Tc cerebral 'metabolic' tracers.

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Single Photon Radiopharmaceuticals for Imaging Myocardial Perfusion

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I. INTRODUCTION

It has long been known that measuring the size of coronary vessels is at best an indirect method of determining tissue perfusion and at worst can grossly misrepresent it. As more sophisticated procedures are developed to treat myocardial ischaemia it becomes more critical to know tissue perfusion,

as opposed to vessel blood flow. Ideally, a measure of the metabolic state of the tissue is needed, however, at present this is beyond the reach of routine nuclear medicine.

There is one major requirement that must be met in order that an injected tracer accurately depicts myocardial perfusion. This is that the extraction of the tracer be high for the range of perfusion that is normally encountered. The resting myocardial blood flow is of the order of 60-80mL/min/100g tissue, however, the heart has an extraordinary dynamic range and blood flow can increase 4 or 5 times during peak exercise or pharmacologic stress. Also desirable is that the input function be short, this is normally achieved concomitant with a rapid blood clearance.The matter of residence times in the myocardium of the extracted radioactivity is much more fluid than in the past as instruments have improved and as the clinical goals have changed.

II. MONOVALENT CATIONS

For over a decade the main means of achieving a high myocardial extraction of a tracer has been to use the efficient transport properties of the sodium-potassium ATPase pump. This pump selectively concentrates the potassium ion in a cell. Obviously it would be most desirable to use a radionuclide of potassium for this purpose, however, although there are clinically useful radionuclides of potassium-^{38}K & ^{43}K- neither has ideal physical properties and both are difficult to make.

The sodium/potassium ATPase pump is not selective for potassium ions *per se* but instead has affinity for monovalent cations of a certain ionic radius. Thus other cations can be taken up by this system although not as efficiently. Of these cations the thallous ion has the highest extraction. ^{201}Tl has adequate, (but less than optimum), production characteristics and physical characteristics to have made it the workhorse of myocardial perfusion imaging for the past decade.

The thallous ion has some deficiencies when it comes to using it to depict myocardial perfusion. The very use of the sodium/potassium ATPase pump to achieve high extraction may lead to misrepresentation of flow. This is because the pump requires metabolic energy to function and thus pump activity may become uncoupled from flow. It has been shown for instance that major changes in the activity of the pump occur during accute ischaemia and following reperfusion.[1] Much of the pharmacology and pharmacokinetics of the thallous ion has been derived from that of the potassium ion with the assumption that the two are very similar. Whilst there are many similarities the differences, which are normally ones of degree, are significant.

The thallous ion is more reactive than the potassium ion and can form complexes with sulphur containing ligands [2] which suggests that a significant portion of the thallous ion injected could be complexed with sulphur containing amino acids at physiological pH. This may be one of the causes of the lower extraction of the thallous ion vs the potassium ion apart from the aknowledged differences in their hydrated ion radius. In a similar vein it is known that the thallous ion forms a neutral complex with the chloride ion at physiological pH. This species is relatively insoluble in aqueous media, however, the solubility product is not exceeded in the clinically used Tl-201 solutions. This neutral species is capable of crossing lipid bilayers to an appreciable extent and so could be extracted by the myocardium independent of the operation of the sodium potassium ATPase pump.[3] There is some evidence that this does indeed occur for example not all of the uptake of the thallous ion can be inhibited by ouabain.[4,5] The kinetics of the thallous ion are also different from those of the potassium ion. In the rabbit isolated septum the uptake and washout of the thallous ion is slower than that of the potassium ion. [6]

The thallous ion is not homogeneously distributed within the cellular compartment within the myocardium but instead exhibits multicompartmental

kinetics.[6] This was also observed in rat myocardial tissue.[7] On the other hand, studies with isolated cells have generally identified only a single compartment within the cells. [8,9] From a clinical point of view the tissue results are more relevant than the cultured cell results and point to the fact that parts of the myocardium other than the myocytes influence the kinetics of the thallous ion. The effects of hypoxia on the uptake and washout of the thallous ion has generally been shown to be small relative to the effects of ischaemia.[9]

The thallous ion is not fixed in the myocardium but is in equilibrium with thallous ion in the blood. This has been clearly demonstrated using isolated myocytes [4] and is a feature common to many cells [5]. This has been turned to advantage for the well known redistribution studies which are performed to separate ischaemia from infarct. Of some concern is the imprecise nature of the kinetics of redistribution. The small amount of thallous ion in the blood is not the sole source of redistributing thallous ion but is in communication with all the tissues in the body.

Some attempts have been made to try and improve on the pharmacokinetics of the thallous ion by using a thallium complex that has a fixed disposition in the body after the initial distribution phase. Such a complex is thallium diethyldithiocarbamate, (TlDDC), which has been investigated as a means of imaging cerebral blood flow.[10] This compound remains fixed in the brain after the initial distribution phase. It is also taken up by the heart [10] and so was investigated to see if it would remain fixed in the myocardium and exhibit no redistribution as does the thallous ion. Studies in isolated rat myocytes and in a limited number of patients showed that washout of thallium occurs in both cases and redistribution of the radioactivity in the myocardium of the patients occurs in a similar fashion to the thallous ion.[11] This is not suprising as the TlDDC complex is weak and breaks down rapidly in vitro and in vivo to release the thallous ion. In the brain this remains

relatively fixed but in the heart it can escape and undergo redistribution.

III. TECHNETIUM AGENTS

The decade of success of ^{201}Tl myocardial imaging lead to the search for a replacement which did not suffer the perceived deficiencies of suboptimal availability, poor physical characteristics and a complex and poorly understood clinical pharmacology. Initially a 'thallium replacement' was desired i.e. a compound that had the same pharmacokinetics as the thallous ion when used in the clinic. Seen from a thallium viewpoint better image quality was an obvious improvement provided by technetium. Such an improvement had already been obtained by the use of SPECT with thallium, so SPECT and new technetium agents was an obvious combination. This line of reasoning defined one of the requirements of a new technetium agent, namely a long residence time in the heart to accomodate the long acquisition time needed by the ubiquitous single headed rotating SPECT cameras. However, image quality is not the only advantage that the new technetium agents might offer. The new technetium compounds may also be designed to have better (different) pharmacokinetics, e.g. a short input function, no washout from the heart washout from the heart in proportion to flow etc. Many of these characteristics are mutually exclusive and so more than one technetium agent is needed.

A. Complex Cations

The desired technetium compounds must be taken up by the myocardium in proportion to blood flow. This requires that they have a high extraction and a slower washout than uptake rate.

The first attempts at making such a compound used as the starting principal the observation that many cations have an affinity for the heart, not just those of the alkali metals.[12] A variety of cationic technetium (III) complexes were made but only the octahedral complexes with o-

phenylenebis(dimethylarsine) (DIARS) were characterised to any great extent (Figure 1).

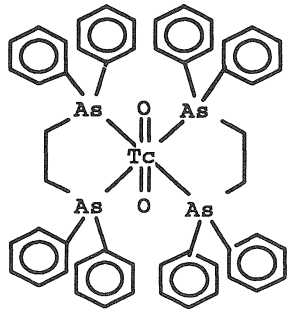
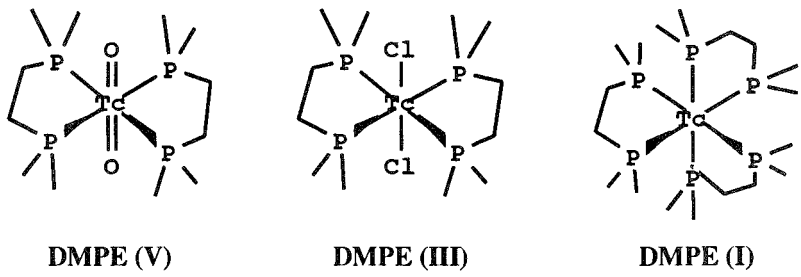


Figure 1. Structure of DIARS

In addition to the DIARS complexes, presumed cationic complexes were made and tested with three other chelating agents - bis(1,2-diphenylarsino)ethane (DAE), tris(2-diphenylphosphinoethyl) phosphine (TETRAPHOS) and Bis(2-diphenylphosphinoethyl)amine (PPN). These other complexes were assumed to have the same structure as the DIARS complexes. This may be true for the DAE complexes but is not necessarily so for TETRAPHOS and PPN as these chelating agents are not necessarily bidentate nor need they have the same geometry as the bidentate DIARS and DAE chelates. Only the DIARS series provided images of the myocardium. The lack of characterisation of these other complexes is unfortunate as the authors ascribed the desirable characteristics of the DIARS complexes to some property afforded by the DIARS itself. This conclusion is only tenable if the structures of all the complexes are identical. Nevertheless the results demonstrated for the first time that it is possible to make a technetium compound that goes to the myocardium in animals.

1. SIMPLE PHOSPHINES

Based upon the conclusions derived from the previous work the same authors made and tested similar complexes using phosphorous rather then arsenic as the ligating atom. The Tc(III) complex with bis(dimethyl phosphino)ethane (DMPE) (Figure 2) exhibited good myocardial uptake in animals [13] and so was examined in man where it gave abysmal myocardial images [14]. The same fate befell the analogous bis(diethylphosphino)ethane (DEPE) complex [15]. The Tc(V)DMPE complex showed poor uptake in the myocardium in animals and no uptake in the myocardium in man [16]. The final member of this series, the Tc(I)DMPE complex, showed uptake in animal and human hearts but suffered from a prolonged blood clearance in man such that clear images of the myocardium could not be obtained until some hours after injection [16,17].



DMPE (V) **DMPE (III)** **DMPE (I)**

Figure 2. Structures of DMPE Complexes

Much time was spent trying to sort out the characteristics of this group of compound in various animal species and finally a reasonable explanation of the mechanisms involved was developed. A lucid description of the work has been published [16]. In brief, transient uptake of radioactivity in the human myocardium occurs after injection of Tc(III)DMPE but trapping was not achieved because of a reductive mechanism that is prominent in the human

heart. This reductive mechanism forms a neutral Tc(II) species, from the original Tc(III) cation, which can escape from the heart. Electrochemical studies of Tc(III)DMPE show that it has a formal reduction potential ($E^{o'}$) of -0.231 volts which is within the biologically accessible range [18]. Significant washout of radioactivity from the myocardium of most animal species suggests that conversion to the neutral species may occur in all animal species, however, the rate is prohibitively fast in man. The influence of this reductive mechanism was elegantly demonstrated by using the Rhenium analogue of Tc(III)DMPE which cannot be reduced and which has a very slow washout from the dog myocardium relative to the Tc compound. The Tc(I)DMPE complex fails in man not because of this reduction mechanism to which it is immune, but because it exhibits exceptionally high protein binding in man.

The results of these experiments with the DMPE series of complexes suggested that reduction of a Tc(III) species to the neutral Tc(II) species must be avoided. The first attempt at doing this by using a Tc(I) species was partially successful in that good myocardial uptake and prolonged retention was achieved, however, as an imaging agent the Tc(I)DMPE complex failed in man because of high blood activity. There were, therefore, two directions that further developments could take -

a) make less easily reducible Tc(III) complexes.

b) make Tc(I) complexes with less blood retention.

Both of these approaches were persued the second of course being a subset of the first. The option of using Tc(V)oxo complexes was not initially persued because none of those previously tried have ever demonstrated myocardial uptake [16], the same generalisation was made of Tc(V)N complexes, however, there has recently been a report of a Tc(V)N(DMPE) complex which does go to the myocardium in small animals [19]. This compound shows an unexpectedly low Tc(III)/(II) reduction potential and as expected washes out of the myocardium rapidly. In addition a Tc(V)oxo species has now been found

that gives good myocardial images in all animals including humans. (see below)

2. Tc(I) COMPLEXES WITH LOW BLOOD RETENTION - PHOSPHITES, ETC.

A second type of Tc(I) complex based on monodentate phosphites, phosphonites or phosphinites, (i.e. containing the P-O-C fragment), has been tested to see if it is taken up and retained by the myocardium but does not suffer from prolonged blood retention. The main compound Tc(I) hexakis(trimethylphosphite) (TMP) has a long inorganic chemistry history [20] (Figure 3). All compounds were taken up and retained by the myocardium when examined in rats, rabbits, dogs, cats and pigs [21]. Imaging studies using TMP in various animals gave good myocardial images soon after injection. Subsequent human studies showed that TMP has the same characteristics and faults as the TBI isonitrile complex (see below), that is, imaging of the myocardium cannot be performed until some hours after injection because of high blood and liver radioactivity. These observations were confirmed by other groups [22].

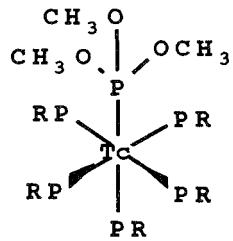


Figure 3. Tc(I)hexakis(trimethylphosphite)(TMP)

Another variation tried was to test bidentate versions of these ligands for instance Tc(I)tris(bisdimethoxyphosphinoethane) (POM-POM) [22], this too gave myocardial images only after obscuring radioactivity in the lungs, liver and blood had cleared. As with the other failures in this class of compounds the

high background radioactivity in humans was not predicted by animal studies.

3. NEWER FUNCTIONALIZED PHOSPHINES

The large amount of negative data collected on the Tc(III/I) phosphorous-based-ligand complexes would suggest that there is some fundamental property of these ligands that makes them unsuitable for myocardial imaging in humans. Recent developments belie such a conclusion as, after extensive testing of a large number of compounds, two have now been shown to possess the desired properties.[23] The compounds tested were 1,2- or 1,3-diphosphines containing different functional groups either on the backbone or as a substituent off the phosphorous atoms. The functional groups included ethers, aldehydes, ketals, acetals, alcohols, amines, alkenes, silanes, thioethers, amides, nitriles and phospholes. Out of this large number and variety of compounds that were made and tested in animal models the two that are successful in man are $Tc(V)(O)_2$ (1,2-bis(di((2-ethoxy)ethyl)phosphino)ethane)$_2$ (P53) and $Tc(I/III)(NO)(Cl)$(1,3-bis(dimethylphosphino)-2,2-di(methoxymethyl) propane)$_2$ (PL37) (Figure 4).

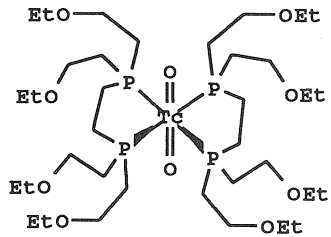


Figure 4. $Tc(V)(O)_2$(1,2-bis(di((2-ethoxy)ethyl)phosphino)ethane$_2$ (P53)

Both of these compounds are characterised by having rapid uptake and long retention by the myocardium with little retention of radioactivity in the background organs. Each of these compounds is interesting and anomalous in

its' own right. P53 is a Tc(V)=O species which until recently had not been shown to concentrate in the myocardium of any species and it has been suggested that this was because of the presence of the polar oxo group. Not only does P53 have the polar oxo group it also has eight polar ethoxy groups per molecule derived from the ligand. Similarily PL37 contains two different axial ligands one of which (NO) makes the assignment of the oxidation state somewhat arbitrary.

4. NON-BIOLOGICALLY REDUCIBLE Tc(III) COMPLEXES - SCHIFF BASES

In order to change the redox potentials of the Tc(III) it is necessary to change the strength of the ligand field around the Technetium atom. This can be achieved by judicious use of a combination of monodentate and multidentate ligands. The most commonly used system has been that of a tetradentate Schiff base coupled with two trans monodentate ligands which in the basic Tc(V) complex are oxo ligands [24]. The oxo groups have been replaced by a variety of phosphorous or isonitrile ligands which reduce the Technetium to Tc(III) [25]. The electrochemistry of these ligands has been extensively analysed and establishes a range for the reduction potential for Tc(III)/(II) of -0.693 to-1.108 volts [26] i.e. much harder to reduce than the corresponding DMPE complexes.

Combinations of two Schiff base ligands, N,N'-ethylenebis(acetylacetone imine) (En) and N,N'-propylene-1,2-bis (acetyl acetone imine) (Pn) and three substituted phosphines - PMe_3 (M),PEt_3 (E) and PMe_2MeOH (MM) were tested in animals and man [27] (Figure 5).

EnM, EnE, EnMM and PnM all exhibited myocardial uptake in rats with an apparent correlation to the lipophilicity of the complexes. All showed slow washout of radioactivity from the myocardium. Similar results were obtained

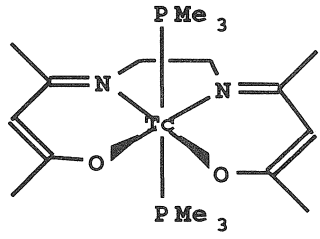


Figure 5. Structure of Schiff Base Ligands

with EnM, EnE and EnMM in dogs. One complex, EnM, was tested in man and provided myocardial images from one to at least five hours after injection. The images were of poor quality because the absolute amount in the myocardium was low and because of prolonged blood retention of radioactivity. More recently a further derivative of this class of compounds, $En((PCH_2CH_2CH_2OCH_3)_3)_2$ has been tested in humans [28] and was shown to have about 2.5% uptake in the myocardium and other 'reasonable' biological characteristics. The reason for the improvement over the similar complexes previously tested was ascribed to the presence of the ether groups.

These results provide additional credence to the view that biologicaly reducible Tc(III) compounds clear from the heart whereas reduction resistant complexes do not. However, many of the compounds were still inadequate because of low myocardial uptake and protein binding. The latter problem is reminiscent of the Tc(I)DMPE complexes and as we shall see the first of the isonitrile based Tc(I) complexes. In each case improvements were made by adding polar groups to the outer dimensions of the ligands. In each case the high protein binding which causes prolonged blood retention in man was not observed in animals.

5. Tc(I) COMPLEXES WITH LOWER BLOOD RETENTION - ISONITRILES

Cationic compounds based on technetium (I) complexes with isonitrile ligands [29] have been made which by default could not fall foul of the reductive mechanism in the human heart because of the low oxidation state of the technetium. The first of the series is shown in Figure 6.

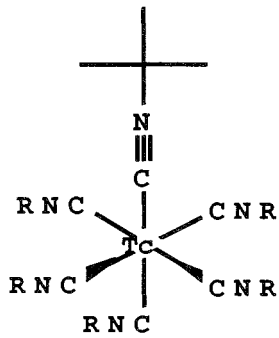


Figure 6. Structure of TBI

A series of these complexes were examined in biological models and some attempt was made to describe the relationship between lipophilicity and the biodistribution. [30]

Table 1. Acronyms Assigned to Isonitrile Complexes by Refs. 30, 32 and 39

ACRONYM	R in figure 6	ACRONYM	R in figure 6
TBI	$CNC(CH_3)_3$	EEG	$CNCH(CH_2CH_3)CO_2CH_2CH_3$
CPI	$CN(CH_3)_2CO_2CH_3$	MEG	$CNCH(CH_2CH_3)CO_2CH_3$
MIBI	$CNCH_2C(CH_3)_2OCH_3$	EGI	$CNCH_2CO_2CH_2CH_3$
MEI	$CHCH(CH_3)CO_2CH_3$	EMAI	$CNC(CH_3)_2CO_2CH_2CH_3$
EEI	$CNCH_2CH_2CO_2CH_2CH_2$	BCPI	$CNCH(CH_3)CH_2CO_2CH_3$
BCNI	$CHCH_2CH(CH_3)CO_2CH_3$	PMI/IPG	$CNCH_2CO_2CH(CH_3)_2$
BPI	$CNC(CH_3)_2CO_2CH_2CH_2CH_2CH_3$	EBA	$CNCH_2CH_2CO_2CH_3$
NPG	$CNCH_2CO_2CH_2CH_2CH_2$	EAI	$CNC(CH_3)_2CO_2CH_2CH_2$

The lipophilicity of the complexes was determined by reversed-phase HPLC using a linear methanol gradient which tends to compress the elution times and so some resolution may have been lost. The lack of a recognisable relationship between lipophilicity and heart uptake in Figure 7 is also a result of the paucity of data. One compound, TBI, does seem to have higher uptake.

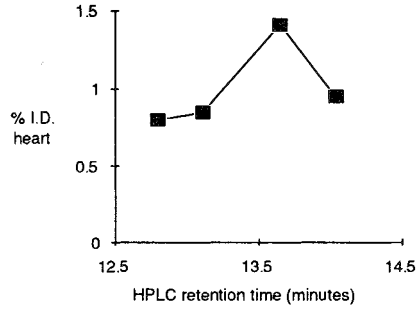


Figure 7. Relationship Between Heart Uptake in Mice at 30 Minutes and the Lipophilicity of Tc(I) Isonitrile Complexes

Similar plots of liver activity in rabbits or the uptake into Chinese hamster V79 lung fibroblasts are also of little predictive value. (Figure 8).

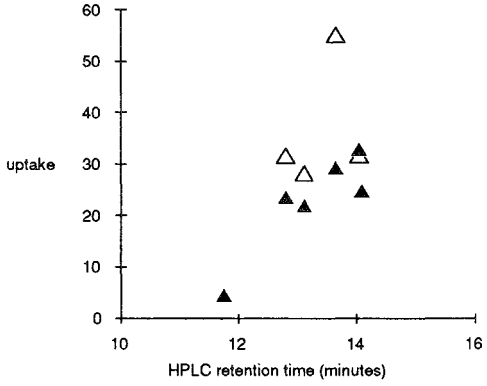


Figure 8. Relationship Between Lipophilicity and the Uptake of Radioactivity in the Liver (Δ) of Mice at 30 Minutes Post Injection and in V79 Cells (▲) after 1 h Incubation.

Based on encouraging heart uptake in rabbits TBI was examined in man and was found to be retained in the human heart with a long half life.[31] It has the drawback that activity initially in the lungs and liver precludes the acquisition of good images until about one hour after injection. Blood clearance is suitably rapid and is not the limiting factor to seeing the myocardium. It may be that the lung/liver uptake and retention is a manifestation of the same phenomenon that cause the blood retention of the other Tc(I) complexes. Certainly it is linked to the lipophilicity of the complex as increased lung uptake was exhibited by Tc(III)DEPE vs the DMPE analogue [16]. Therefore, a series of less lipophilic, predominantly ester derivatives of the isonitrile complexes was developed. The rationale was to try to reduce liver radioactivity by introducing a cleavable ester group that once metabolised would produce a less lipophilic complex. This complex would be preferentially excreted by the renal system and, if taken up by the liver would pass rapidly through it.

A range of eight isonitrile complexes MEI, NCPI, EEI, CPI, PMI, MIBI, TBI, BPI have been examined in cultured chick myocyte/rabbit imaging models to explore some of these points [32]. The lipophilicity of the complexes was determined by reversed-phase HPLC using the same linear methanol gradient as before.(Table 2).

Table 2. Lipophilicity and Uptake Data for a Series of Isonitrile Complexes

Acronym	HPLC	clog P	cell	heart
MEI	8	-0.283	20	129
NCPI	9	-	24	19
EEI	8.6	0.301	28	137
CPI	9	0.026	100	243
PMI	9.1	0.246	349	167
MIBI	10.1	0.235	168	340
TBI	10.1	0.843	910	77
BPI	12.2	1.613	104	77

The authors compared the lipophilicity and the uptake data and found a reasonable linear correlation (r=0.71). They noted some anomalies, i.e. EEI, PMI and CPI have similar lipophilicities as measured by HPLC but have dissimilar cell and heart uptakes. This they suggested was due to the different stereochemistries of the complexes. In addition they noted that BPI was the most lipophilic of the complexes but did not have the highest uptake as predicted by the linear correlation.

When these data are presented graphically, (Figure 9), the validity of a linear relationship between lipophilicity and uptake is not convincing and the relationship between EEI, PMI and CPI does not look unusual.

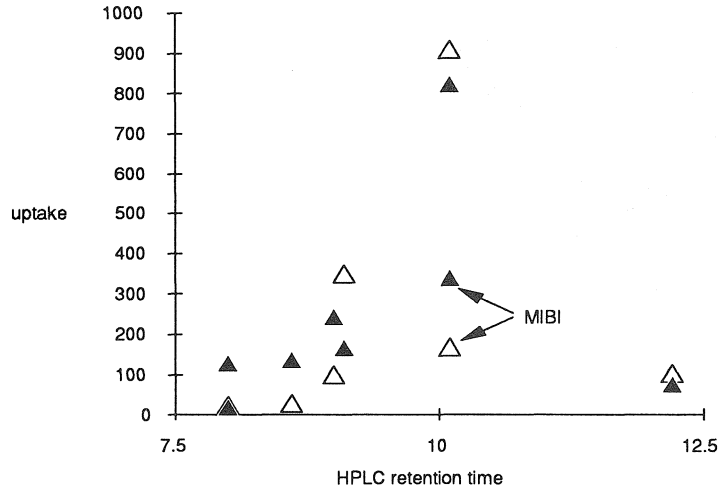


Figure 9. Relationship Between Cell (Δ) or Myocardial (▲) Uptake and HPLC Retention Time of Isonitrile Complexes

The existence of a parabolic relationship between lipophilicity and a whole range of biological properties is well documented.[33,34] Such a parabolic relationship has been demonstrated for at least three classes of

Technetium based myocardial perfusion imaging agents [21,35,36] and it is possible that these isonitrile data are part of a parabolic set. Whether linear or parabolic the position of MIBI, which happens to be the compound which was selected for commercialisation, appears to be anomalous. MIBI is the only ether in the data set and may have some specific interaction with the HPLC system which has returned an erroneous lipophilicity for MIBI.

One way to get over this is to calculate the lipophilicity of the complexes. When comparing a data set it is not necessary to know the absolute lipophilicity but instead the relative lipophilicity will suffice. If one assumes that the chosen core has a constant contribution and interacts in a simple additive manner with the changing part of the molecule one can derive the lipophilicity and compare only the changing part of the molecule. This is much simpler than trying to derive the contribution of the technetium atom. Such a procedure has been done before with Technetium complexes.[37,38] The results, obtained using Medchem software, are recorded as clogP values in Table 2. clogP is the calculated log P of the compounds formed when the isonitrile group is replaced by a nitrile group. (This was done as Medchem does not have a fragmentary constant for the isonitrile group itself). The figure for NCPI is missing as this complex is made up of five CPI ligands and one deesterified CPI. It is in principle easy to determine the adjusted log P of such a complex, however, it is not so easy to accomodate the fact that it is formally a zwitterion not a cationic species. When this measure of lipophilicity is plotted against uptake the resulting graph does not contain MIBI in an anomalous position (Figure 10). Transformed in this way the data in Figure 9 now look much less like linear data and more amenable to a parabolic fit with the peak near TBI and with the low uptake of BPI explainable by it being 'over the top'. No conclusions can be drawn about the relationships between EEI, PMI and CPI.

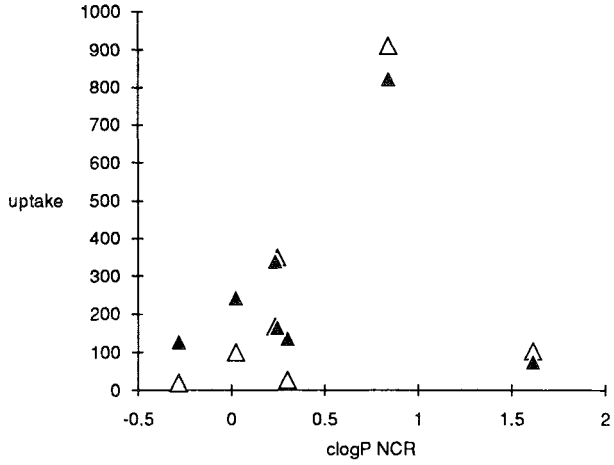


Figure 10. Relationship Between ClogP and Cell (Δ) or Myocardial (▲) Uptake of Isonitrile Complexes

The validity of using clogP values of a fragment of the complex rather than using log P values of the complexes as determined by the traditional shake flask method or by HPLC has been demonstrated before for other types of technetium complexes [37] but is also demonstrated for the isonitriles in Figure 11. Slightly different HPLC systems were used for the two data sets yet the slope of the two regression lines is virtually the same. The data for the more homogeneous alkyl derivatives shown by the open triangles is much better behaved than that of the other data set, however, the relationship is predictive for both. The calculation of the lipophilicity rather than the measurement of it has the potential for increasing the development rate and reducing the cost of such compounds.

The influence of lipophilicity may be stronger than these authors believe and their suggestions on the possible effects of stereochemistry insufficiently separated from lipophilicity such that they may be different descriptors of the same phenomenon.

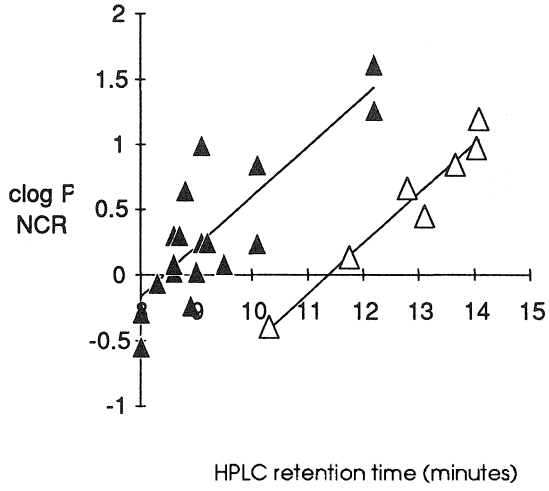


Figure 11. Relationship Between a Calculated Lipophilicity of a Compound and its Lipophilicity as Determined by HPLC.

All except one of the second generation compounds incorporates ester groups which upon cleavage are designed to produce rapidly clearing (more hydrophilic) species. (The exception is MIBI which contains an ether group.) Base hydrolysis of Tc-99 material followed by NMR analysis produces signals of each of the obvious species expected from deesterification of from 1-6 groups to form ultimately the pentanegative species (Table 3). The di-, tri- and tetra-hydrolised species also exhibit isomers. The acid isonitriles have a pKa of 2.95, in the normal region for aliphatic acids [39].

Analysis of the initial rate of hydrolysis of a variety of ester containing complexes by rat, rabbit or human plasma demonstrated a wide range of rates but also some order. The rate of hydrolysis is species dependent and follows the order rat>>human≥rabbit.

Table 3. Hydrolysis of Different Isonitrile Esters by Plasma From Various Species Incubated at 37°C for Two Minutes (Expressed as % Tc)

Ester	Rat	Rabbit	Human
NPG	400	366	400
EAI	300	64	173
BCNI	265	6	28
EEG	254	20	119
MEG	234	45	144
EGI	209	81	87
EMAI	207	0	43
CPI	200	65	80
BCPI	185	15	30
IPG	150	0	0
EBA	131	2	14

The slope of the data in Figure 12 is approximately unity and the major difference is that the rabbit and human curves appear to be offset by 130%. The correspondence between the rabbit and human data is good but a few compounds, MEG, EEG, EAI, that possess a single substituent alpha to the isonitrile group stand out as being different. This suggests that there are some subtle as well as gross differences in the characteristics of the esterases from the different species. Indeed further studies comparing hydrolysis rates of CPI by rat, mouse and human plasma established that the activation energy of the rodent system is 10% lower than the human system, the rat system can only hydrolyse two of the ester functions out of the six on each complex and finally the distribution of isomers is different for the di-hydrolysed species [39]. Biodistribution studies of the hydrolysed species clearly demonstrated major differences to the starting complex and confirmed the original hypothesis that liver clearance would be enhanced.

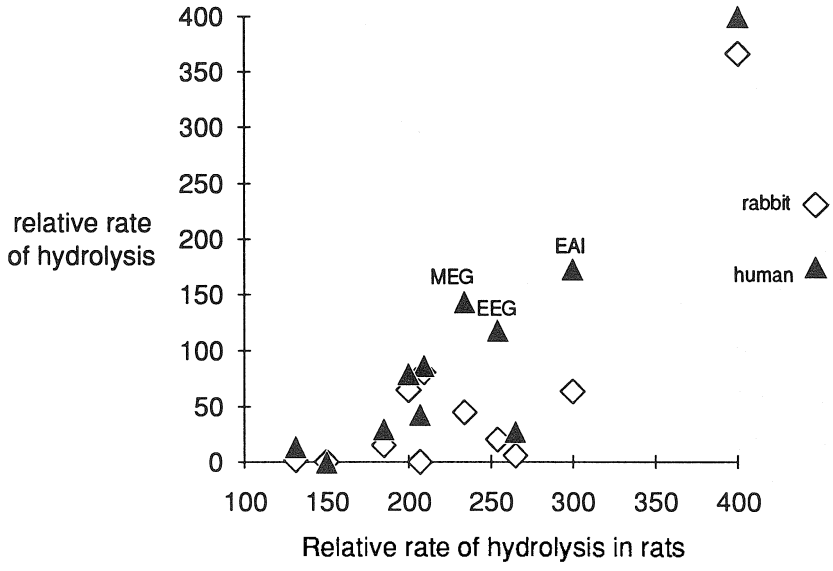


Figure 12. Relationship Between Rat, Rabbit and Human Hydrolysis Rates

6. Tc(I) COMPLEXES WITH LOWER BLOOD RETENTION - ARENES

A final and somewhat unusual attempt to find a useful series of Technetium cations for myocardial imaging was based upon well known organometallic chemistry principles. In a belief that the size of the cation played a major role influencing myocardial uptake a series of small cationic Tc(I) complexes was developed based upon diarene complexes.[21] These are sandwich compounds in which the Technetium atom lies between two coplanar benzene ring (Figure 13).

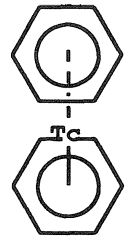


Figure 13. Structure - Arenes

The preparation of the compounds is quite different to that of all other potential radiopharmaceuticals being from pertechnetate heated in an aromatic solvent in the presence of aluminium catalysts. A large number of derivatives were made and tested in animals and showed promising myocardial uptake. The structure and method of preparation of these compounds allowed a large homologous series to be tested. Convincing QSDRs were generated based mainly on the number of methyl groups which was predictive of the lipophilicity of the complex. Much better correlations can be generated if one uses log P of the aromatic groups as the independent variable. This is convenient as many of them have been measured by the shake flask method but they are also very amenable to calculation by Medchem (Table 4).

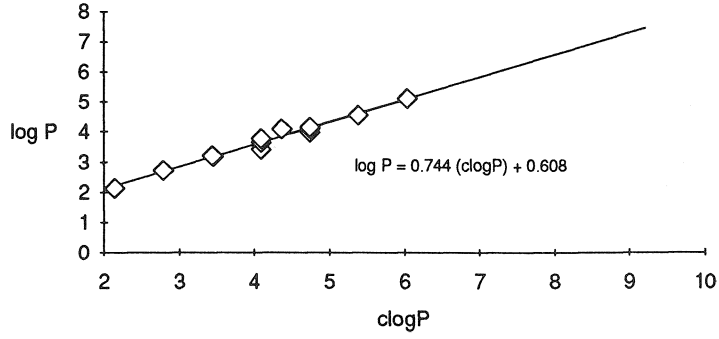


Figure 14. Relationship Between ClogP and Measured Log P for the Aromatic Functions of the Tc Arenes

Not all of the measured log Ps of the aromatic groups were available, those that were not, (* in Table 4), were calculated from the relationship

between clogP and log P as shown in Figure 14.

Table 4. Data on Tc(I) Arenes

number	clogP	log P	heart	prot. bind.
1	2.14	2.13	0.18	11
2	2.79	2.73	0.22	11
18	3.46	3.18	1.07	17
3	3.44	3.20	0.47	11
5	4.09	3.42	1.76	19
4	4.09	3.66	1.01	24
6	4.09	3.78	1.63	21
9	4.74	4.00	2.35	42
12	4.37	4.10	2.50	75
7	4.74	4.11	2.52	47
8	4.74	4.17	3.48	36
13	5.39	4.56	3.39	56
14	6.04	5.11	3.75	74
10*	4.50	3.95	2.79	68
11*	4.50	3.95	3.16	61
15*	5.68	4.83	2.89	83
16*	8.07	6.61	1.72	81
17*	9.21	7.46	1.30	84

These log P values were then used as the independent variable in plots of lipophilicity vs human plasma binding and myocardial uptake in rats at five minutes (Figures 15, 16).

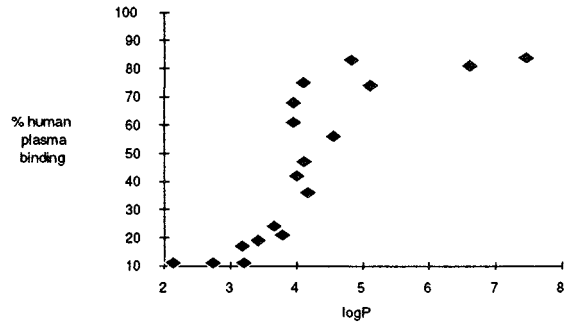


Figure 15. Relationship Between Lipophilicity and Human Plasma Protein Binding of Tc(I) Arene Complexes

An excellent sigmoidal relationship is obtained between lipophilicity and human plasma binding. Myocardial uptake is parabolic vs lipophilicity as we have come to expect (Figure 16).

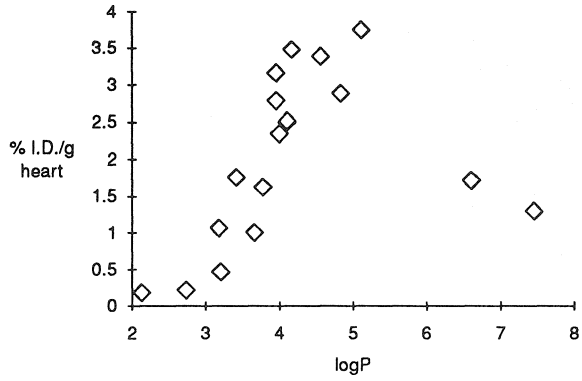


Figure 16. Relationship Between Lipophilicity and Myocardial Uptake in Rats at Five Minutes

At least one of these complexes was tested in dogs and provided good images of the myocardium from 5-60 minutes after injection [21]. There appears to be little washout from the heart and negligible lung or blood retention. The major route of excretion seems to be the kidneys and there is not much radioactivity in the liver. Unfortunately when these compounds were taken into man they proved disappointing with low myocardial uptake and high liver uptake [40]. Once again the animal data did not predict the human condition.

In summary although there have been many attempts to develop nonreducible Tc(III) complexes or Tc(I) complexes with low background radioactivity only the oxygen containing isonitriles or phosphines have been successful. In all cases the failures in man were not predicted by the results of

animal testing.

B. Neutral Species

The final group of players on the stage of myocardial perfusion imaging agents is the BATOs.[41] (Figure 17). Unlike all the other agents mentioned so far these compounds are not monovalent cations but are instead neutral. They are further distanced from the other technetium based compounds in that the ligand is not present in the vial before addition of pertechnetate, (it is formed by template synthesis around the technetium). The building blocks of the ligand are three dioxime molecules, an axial ligand and a capping boronic acid derivative. The final complex consists of dioxime ligands bound to a seven coordinate technetium atom. This in itself is somewhat unusual as is the fact that the dioxime ligands are capped at only one end by a boronic acid group.

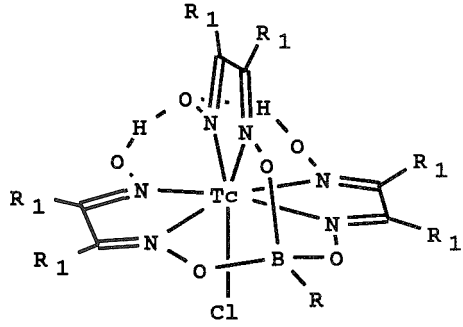


Figure 17. Structure of BATOs

The method of synthesis of the final BATO complex is quite simple and because it is one of template synthesis allows a wide variety of compounds to be made relatively easily. The variety is achieved by combinations of boronic acid and dioximes precursors, technically complex organic synthesis is

avoided. The lipophilicity of many of the complexes is high and as the complexes were not always available at the high purities needed for the 'shake flask' method the lipophilicity of the complexes was measured in relative terms by using the HPLC retention time (log k'). The HPLC system was calibrated to yield log P if required. As data were collected it became apparent that the fragmentary approach could be applied to the BATOs to some advantage. Thus, assuming the core of the complex is constant the variable portion of the metal complex can be treated as a substituent with its' own log P, designated log P(R) (Figure 18). [42]

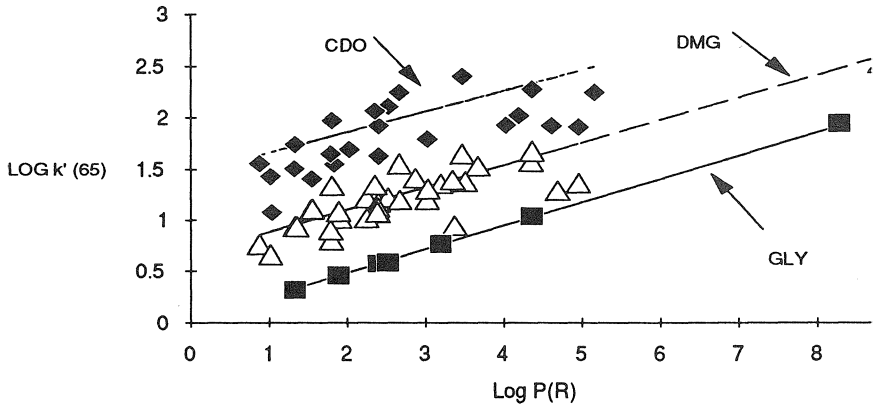


Figure 18. Relationship Between Measured Lipophilicity (HPLC) and Fragmentary Lipophilicity of Boron R Group of BATOs

After testing over 50 different compounds in rats some useful QSDRs were developed for cyclohexane (CDO) or dione dioxime dimethyl glyoxime (DMG) based BATOs having no ionisable groups [35]. At five minutes radioactivity in the lung and liver can be predicted by a linear relationship from the lipophilicity. (Figures 19, 20)

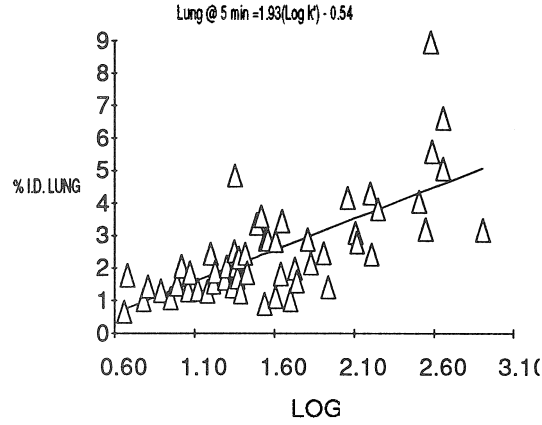


Figure 19. Lung Radioactivity in Rats at Five Minutes vs. Lipophilicity as Determined by HPLC

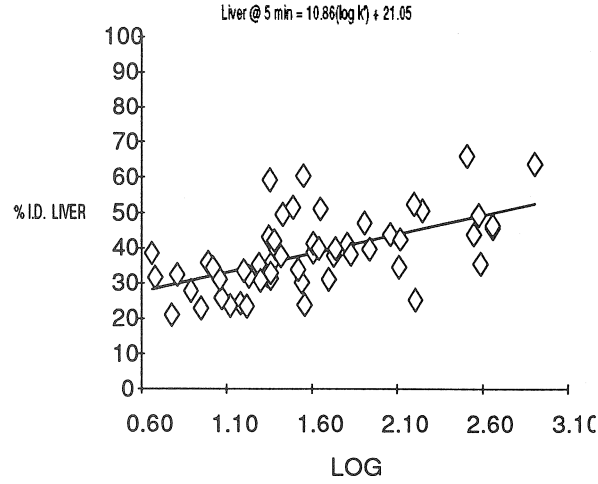


Figure 20. Liver Radioactivity in Rats at Five Minutes vs. Lipophilicity as Measured by HPLC

One would expect each of these relationships to become parabolic if compounds with sufficiently high lipophilicity were tested. The heart does

indeed show a parabolic relationship (Figure 21).

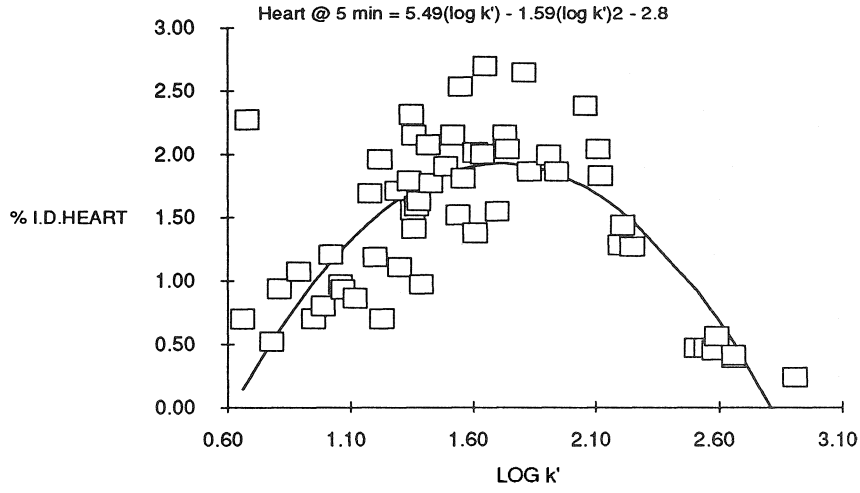


Figure 21. Heart Radioactivity in Rats vs Lipophilicity as Measured by HPLC.

No relationship could be found between lipophilicity and the blood radioactivity at five minutes because the blood levels at five minutes were so low irrespective of the lipophilicity of the BATO. One of the characteristics of the BATOs is that they are rapidly and efficiently cleared from the blood.

A smaller number of compounds was tested in larger animals and imaging studies were done [43,44] in all cases there appeared to be no species differences. Based on these results two compounds were selected and taken into man they were CDO-MeB and CDO-BOH - differing only in the placement of a methyl or -OH group on the boronic acid (R in Fig. 17). The second of these showed a major species difference and did not have the desired characteristics in man, excessive blood retention was observed with little heart uptake, (not the same problem as the cations). The CDO-MeB did live up to

expectations and is being persued as a worthwhile myocardial perfusion imaging agent. [45]

IV. ISOLATED CELL STUDIES

One aspect of these data that is noteworthy is the extraordinary concurrence between the cultured myocyte data and the data derived from whole animal preparations (Figure 9). Such concurrence was not obtained when the cells used were not myocytes [30]. This appears to say that cultured chick myocytes are predictive of the myocardial uptake of a compound in rabbits which suggests that they can be used as a rapid and relatively cheap screening method. This has of course been explored by a number of groups with both chick and neonatal rat myocytes. Extensive use has been made of isolated myocytes to explore the uptake mechanisms of the technetium compounds. This technique has been used to show that the uptake of thallous ion by neonatal rat myocytes was similar to that of K^+ in being via the sodium/potassium ATPase pump. The uptake of Tc(III)DMPE was deemed to be not via the pump because uptake could not be blocked by these ions or by oubain. The rate and magnitude of uptake of the technetium compound was greater than that of Tl^+ or K^+.[4] It has been suggested that results such as these from rat myocytes are fruitless because of the existence of the species specific reduction mechanism for the Tc(III)DMPE complexes [16]. This is an overly pessimistic point of view for two reasons. The first is that the reduction mechanism most likely exists in all species as can be inferred from the fact that Tc(III)DMPE washes out of the myocardium of all species whereas Tc(I)DMPE does not. The second reason is that the mechanism of uptake can still be explored by such studies even if the washout rates are different. Thus the conclusion [4] that the uptake of Tc(III)DMPE is by a mechanism other than that of the sodium/potassium ATPase pump is a valid and useful observation.

TBI has also been examined in neonatal rat myocytes and found to have similar characteristics to Tc(III)DMPE in that it did not appear to be taken up by the sodium/potassium ATPase pump.[46] Unlike Tc(III)DMPE the TBI radioactivity that was bound to the cell was found to be mainly associated with the membrane (78%).

CPI was examined in chick myocytes and found to behave very similarily to TBI in rat myocytes. It was not taken up by the sodium/potassium ATPase pump, (because uptake could not be inhibited by ouabain), and it was also mainly associated with the cell membrane (61%).[47]

In studies in rat myocytes it was found that uptake and release of MIBI was slower than that of the thallous ion and that it was less sensitive to a wide range of metabolic inhibitors. like the other technetium compounds it is probably not taken up by the heart by the sodium/potassium ATPase pump. Impaired contractility was found not to effect the uptake of MIBI (or the thallous ion).[48] The subcellular localisation of MIBI is not the same as that for the other isonitriles and is much more like that of Tc(III)DMPE in that it is mainly associated with the cytosolic fraction.[49] (Table 5) Like the thallous ion some 80% of the radioactivity of MIBI is associated with the cytosolic fraction after differential centrifugation of the cell components. This is true in normoxic conditions both in vivo and ex vivo. Severe hypoxia changes the distribution of MIBI but not that of the thallous ion, the MIBI moves from the cytosol and becomes associated with the mitochondria and the membrane fraction. There are a number of interesting points to consider. First, why is MIBI different to all the other isonitriles in that it concentrates in the cytosol? Lipophilicity is probably not the reason as MIBI has a lipophilicity intermediate to that of TBI and CPI both of which concentrate in the membrane fraction (Table 1). CPI can be rapidly metabolised to a neutral or anionic species but metabolism of TBI is expected to be slow yet they both end up in the membrane. Metabolism of MIBI by hepatic microsomes has

been shown to be very slow [49] yet it does not behave the same as TBI. With the current lack of data one is forced to believe that it is a peculiarity of the structure of MIBI which causes the different localisation, (MIBI is the only ether for which data have been published). The second interesting point is why does the distribution of MIBI change under hypoxia? Thirdly, why is the distribution of MIBI similar to that of Tc(III)DMPE, is it that MIBI is metabolised in myocytes in an analogous fashion to the phosphine?

Table 5. Results of Subcellular Fractionation Studies of Isonitriles

compound	membrane	mitochondria	microsomes	cytosol	ref.
TBI	78				46
CPI	61	9	2	25	47
MIBI	7	6	3	84	49
MIBI(anoxic)	15	35	3	42	49

The BATOs have been examined using both rat and chick myocytes. In the rat cells CDO-MeB was taken up very rapidly, faster than the thallous ion and much faster than MIBI. The washout of CDO-MeB from the cells was slower than that of the thallous ion but faster than that of MIBI. Uptake of CDO-MeB was four times higher than for the thallous ion or for MIBI. There was little effect of various metabolic inhibitors on the uptake of CDO-MeB [50] suggesting that uptake was not mediated by the sodium/potassium ATPase pump, as with the isonitriles. In the chick cells CDO-MeB was shown to have the same four fold increase in uptake over the thallous ion and MIBI, however, some sensitivity to metabolic inhibitors was noted which was similar to that of MIBI.[51]

Thus, overall, the isonitriles and the BATOs are similar in their behaviour with myocytes in that uptake is not inhibited to a great extent by metabolic inhibitors and is not thought to be mediated by the sodium/potassium ATPase

pump. The two classes of compounds are different when the rate and extent of uptake is compared, CDO-MeB is taken up much more rapidly than MIBI and to a four fold greater extent.

V. ISOLATED HEART STUDIES

The extraction of some of these compounds over a range of flows has been examined using isolated buffer perfused rat or guinea pig hearts and blood perfused rabbit hearts. Flow rates were of the order of 10-12 mL/min./g for the rat hearts, 5.5 mL/min./g for the rabbit hearts and, because of the greater oxygen carrying capacity of blood, 1 mL/min./g for the blood perfused rabbit hearts. The thallous ion was 30-40% extracted by buffer perfused rabbit or guinea pig hearts compared to 5% for the intravascular marker albumin. [52] The extraction of Tc(III)DMPE, Tc(I)DMPE, TBI and CDO-MeB can be ranked in the order Tc(III)<Tc(I)≤TBI<CDO-MeB (Table 6).

Table 6. Percent Extraction of Various Compounds by Isolated Perfused Hearts

Species	HSA	Tl	Tc(III)DMPE	Tc(I)DMPE	TBI	MIBI	CDO-MeB	Ref.
Rat			15	33	80			53
Guinea Pig	5	35	5	50	,95	,40		52,55
Rabbit	5	35,60	24	45	100	,35		52,54
Rabbit(blood)		,62			80	45	,75	54,56

When the extractions are compared after simultaneous injection of pairs of compounds CDO-MeB is always more highly extracted than the thallous ion and the thallous ion is always more highly extracted than MIBI. These data agree somewhat with the cultured cell results in that the extraction (or uptake) of MIBI is less than that for the thallous ion or for CDO-MeB. It is crucial that the requirement that extraction be high in order to measure organ blood flow is met for the myocardium as it has such a large dynamic range.

VI. IMAGING CHARACTERISTICS

The kinetics of some of these compounds have been explored using animals. Twenty three different ester isonitriles were examined in rabbits and found to have widely different biodistributions both in where the radioactivity went and in how long it stayed there.[39] The compound with the best overall characteristics selected from high myocardial activity, high renal, low lung, low liver with rapid transit and rapid blood clearance was found to be CPI.

Animal data on MIBI have not been so forthcoming yet it clearly must have been compared to the other isonitriles and shown to be superior. Studies in dogs show that washout of radioactivity from the myocardium is slow, estimates of the rate show that 10-15% of the original activity is washed out in four hours in anaesthetised animals. Radioactivity remaining in the blood at four hours was 2-3%.[57] Radioactivity was distributed in the myocardium in a linear fashion with respect to flow as measured by microspheres.[58]

CDO-MeB has been examined in a variety of animal species and been shown to behave in a similar manner in all.[59,60,61] The blood clearance is fast and more than 90% of the injected radioactivity is cleared from the blood within three minutes after injection.[61] This provides a short sharp input function. Myocardial uptake is rapid and is of the order of three to five percent. Washout from the myocardium is biphasic , the two components are initially present in a 2:1 ratio with t1/2s of 1-4 minutes and about one hour respectively. There is no appreciable lung uptake after the first 1-2 minutes. The major route of excretion is the liver, as for MIBI.

Of all the above isonitrile and BATO compounds two, MIBI and CDO-MeB have been extensively tested in man [62,45] and are well on their way to commercialisation under the trade names Cardiolite(R) and CardioTec(R) respectively. In general the behaviour of these compounds in man is similar to that found in animals. The better myocardial extraction of CDO-MeB over MIBI found in isolated perfused hearts and uptake in cultured chick or rat

myocytes is reflected in man as a higher percent injected dose in the myocardium at five minutes for CDO-MeB compared to MIBI (2.2% vs 1.2%). As in animals the washout of MIBI from the myocardium is slow being of the order of seven hours in man.[62] The washout of CDO-MeB from the myocardium is again biphasic in man with half lives of the order of 6-10 minutes (which may be flow dependent) and some hours.[45] Both PL37 and P53 have been tested in man. They are each characterised by rapid uptake into the heart and rapid clearance of background activity. Washout from the myocardium appears to be prolonged and similar to MIBI rather than to CDO-MeB.

As neither MIBI, PL37/P53 nor CDO-MeB show redistribution of the injected radioactivity in the thallous ion sense images of the myocardium in the resting state must be obtained by a second injection of radioactivity at rest. The slow washout of radioactivity from the myocardium and the apparent lack of any appreciable redistribution after injection of MIBI suggest that a similar image of the myocardium should be obtained for some hours after injection. This has been demonstrated in dogs and has been suggested to be an advantage in some clinical situations.[62] Both SPECT and planar images can be obtained.

In man it has been found expedient to refrain from imaging until about one hour after injection of MIBI, (for both the rest and stress injections), to allow radioactivity to clear from the background tissues, mainly the liver and lungs.[62]

PL37 and P53 have myocardial kinetics similar to MIBI and so can, like MIBI produce images in the heart for some hours after injection. These compounds are different to MIBI in that they are reported to clear more rapidly from the background tissue. This may translate into an ability to image the myocardium sooner after injection than the one hour which appears to be the norm for MIBI. Whether this is clinically relevant remains to be seen.

The relatively fast washout from the myocardium of the majority of the radioactivity after injection of CDO-MeB allows the second (resting) injection to be performed at about 1-2 hours.[45,59,61] Both SPECT and planar images can be obtained. Despite the relatively fast washout of the first component of CDO-MeB, SPECT images of a surgically produced infarct were clearly seen in dogs.[61] This is because the average washout during the acquisition time is sufficiently long not to cause artefacts in the SPECT reconstruction.[63] Similar results have been obtained in man.

VII. THALLIUM VS. TECHNETIUM

Although Tl-201 has been used for over a decade as the workhorse for myocardial imaging the defficiencies of both the thallous ion (low extraction, more than one uptake mechanism, dynamic distribution) and Tl-201 (low energy, long half life, accelerator produced) are such that its' replacement is worthwhile. There is no doubt that a technetium labelled compound would remove the physical defficiencies of Tl-201. There is also no doubt that an alternative for the thallous ion is needed. The two candidates furthest along for the replacement of Tl-201 - MIBI and CDO-MeB - each have quite different in vivo characteristics to the thallous ion.

A superficial analysis of the biological characteristics of the agents might suggest that the thallous ion and MIBI are closely related. They are not. It is true that these two agents go to the myocardium and stay there for a considerable period of time, however the distribution of the thallous ion is constantly changing as it moves towards equilibrium whereas that of MIBI is reported to be essentially fixed.

CDO-MeB is highly extracted by the myocardium but does not stay there for a long time. It has some similarities to xenon in this respect and it may be possible to derive additional perfusion related data from regional washout curves.

Thus the three new Technetium agents for myocardial imaging are only distantly related to Tl-201 and should be thought of as alternatives each with different properties rather than substitutes with very similar properties. These alternatives will not be used in the same way as Tl-201, they each represent new technology and their use will have to be learned.

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4
Iodine Labeled Brain Perfusion Imaging Agents

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I. INTRODUCTION

Because of its potential clinical importance, measurement of cerebral blood flow has been a subject of scientific research for the past half-century (1,2). Recent interest has been centered on developing a suitable gamma-emitting tracer for measuring regional cerebral blood flow (rCBF). In conjunction with positron emission tomography (PET) or single photon emission computed tomography (SPECT), these agents can provide a useful tool for the assessment of rCBF in both normal and disease states.

A. Kety-Schmidt Equation

Measurement of cerebral blood flow using a nonmetabolized diffusible tracer, nitrous oxide, was first reported by Kety (1). This technique employs the "Kety-Schmidt equation":

$$dQ_b/dt = F(C_a - C_v)$$

where Q_b = Quantity of tracer in brain

C_a = Concentration of tracer in arterial blood

C_v = Concentration of tracer in cerebral venous blood

F = Total Cerebral blood flow.

The cerebral blood flow (F) can be calculated by inhaling a constant partial pressure of nitrous oxide until equilibrium is achieved, and by measuring the concentrations of the tracer in arterial (C_a) and mixed cerebral venous blood (C_v), and the amount taken up by the whole brain (Qb).

For small individual regions of the brain, it is necessary to substitute the tracer with a radioactive agent, which is freely diffusible and cannot be metabolized. The quantity in each individual region is measured by autoradiographic techniques in animals, and by external autoradiographic techniques, such as PET and SPECT, in humans. Under appropriate conditions it would be possible to derive the concentration of tracer in the venous blood from a small cerebral region through its tissue concentration. Landau and

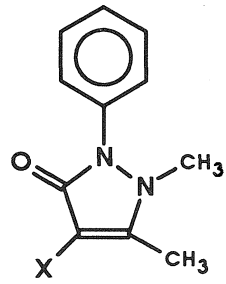
Sokoloff (3) reported the blood flow in various structures in the brain of the cat by using [^{131}I]CF_3I,trifluoromethyliodide, as the tracer. The tracer was injected intravenously in solution and at the end of one minute, the animal was sacrificed and the brain frozen in liquid nitrogen. The tissue concentration in each region was measured by autoradiography. However, there are several drawbacks associated with this tracer. The tracer is a gas at room temperature; therefore, the autoradiogram had to be obtained at -40^{o}C and the iodine-131 contains high energy beta rays which degraded the resolution of the autoradiograms.

In 1966 Lassen reported the use of Xe-133 (gamma ray energy 80 kev) as a tracer for measuring rCBF in humans (4). This tracer is introduced by intracarotid injection and the cerebral blood flow calculated by analyzing the slope of the washout curve. A less invasive procedure is achieved by inhalation of Xe-133 while monitoring the radioactivity in each specific region of interest (5,6). The washout curve is analyzed to obtain the regional blood flow. This relatively noninvasive method has been applied in various neurological disorders. However, this technique is prone to background interference, from radioactivity in other parts of brain and in the respiratory tract.

B. Antipyrines

Improvement of the above mentioned rCBF measuring techniques has been achieved by less volatile tracers labeled with isotopes emitting lower energy beta rays. Reivich et al reported the use of ^{14}C-antipyrine (1,5-dimethyl-2-phenyl-3-pyrazolone) for autoradiography study in animals (7); Sakurada et al (8) employed 4-iodo-^{14}C-antipyrine (Fig. 1). The latter compound, with its excellent capillary permeability, appeared to be the agent of choice for autoradiographic studies of regional cerebral perfusion. The halogen derivatives of antipyrine (4-iodoantipyrine , IAP; 4-bromoantipyrine,

BrAP; 4-fluoroantipyrine, FAP) are generally lipid soluble and can cross the cell membrane by a simple diffusion mechanism. Iodine-123 IAP (9), Br-82 BrAP (10), and F-18 FAP (11,12) have been reported as tracers for measuring rCBF using autoradiographic or imaging techniques (13,14). Major difficulties in using these agents for imaging rCBF in humans is the fast washout from the brain. This problem is especially crucial in SPECT, which traditionally requires 30-60 min data accumulation time with the common single headed gamma camera units.



X	
H	antipyrine
F	4-Fluoroantipyrine
Br	4-Bromoantipyrine
I	4-Iodoantipyrine

Figure 1. Chemical Structures of Antipyrine and Its Derivatives

The current status of SPECT instrumentation places stringent requirements on the biological properties of tracers in order to be useful in the measurement of rCBF. In addition to high uptake in brain, the tracer has to remain fixed for a period of time (30-60 min) to allow for adequate data collection. The emphasis of tracer development centers on the evaluation of agents with high initial brain uptake, fixed regional cerebral distribution (reflecting blood flow) and minimal in vivo metabolism (15). In 1976 it was reported that C-11

alkylamines showed high uptake and retention in both the lungs and the brain (16). It was suggested that these agents were trapped by amine metabolism (either by monoamine oxidase, MAO, or by mixed function oxidase, MFO) and that they may be useful as imaging agents to evaluate lung metabolism. Independently, another group of amines, iodinated catecholamine congeners, 4-1-DPIA (Fig. 2), showing high brain uptake and prolonged retention, were reported (17,18). The design of these molecules was based on their structural similarities to dopamine, mescaline and other psychotomimetic agents (Fig. 2). Therefore, by default, it is assumed that these agents and, later, the iodo-amphetamines (IMP, developed by Medi-physics) (19,20), are neurotransmitter analogs with receptor binding properties (see discussion below).

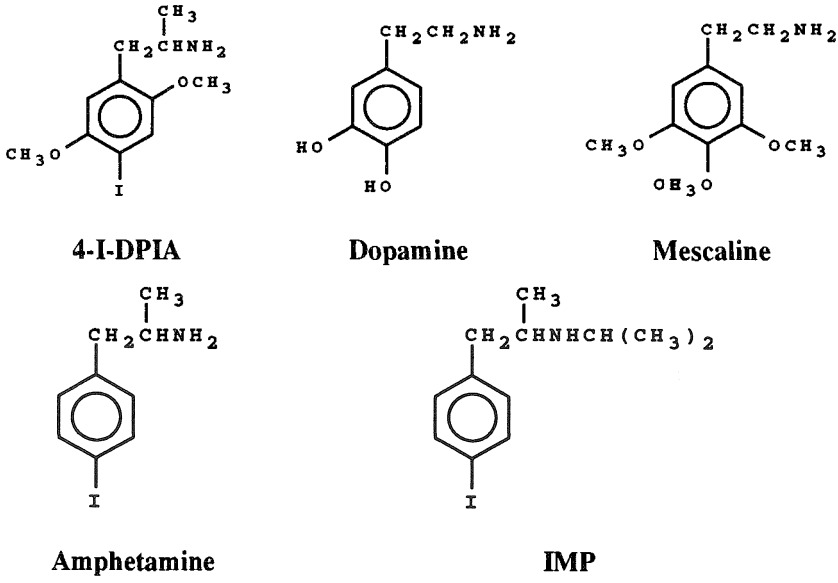


Figure 2. Chemical Structures of 4-1 DPIA, IMP and Related Amines

In 1980, a series of Se-75 diamines (MOSE , PIPSE) was reported (21,22) (Fig. 3). The pH shift mechanism was proposed to explain their brain

uptake and retention (21). Later, based on the same hypothesis, an iodinated diamine, HIPDM, was developed as a brain perfusion imaging agent (23,24) (Fig. 3).

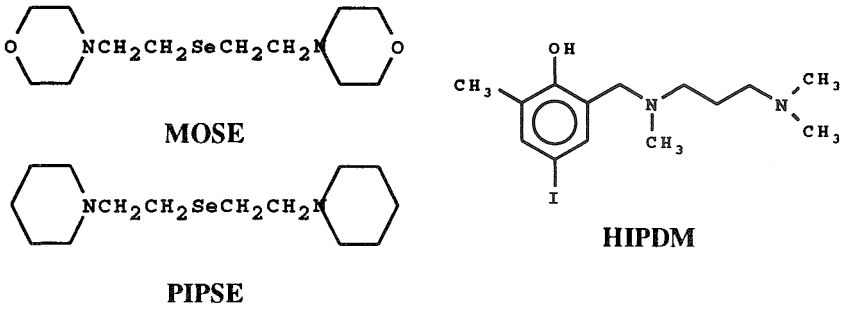


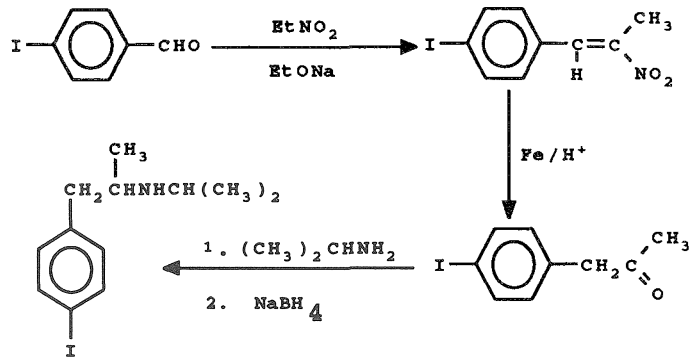
Figure 3. Chemical Structures of MOSE, PIPSE and HIPDM

Until now, most of the clinical evaluations have been conducted using either IMP or HIPDM; therefore, the remainder of this chapter has been devoted to the discussion of the chemistry, pharmacology and clinical applications of these two agents.

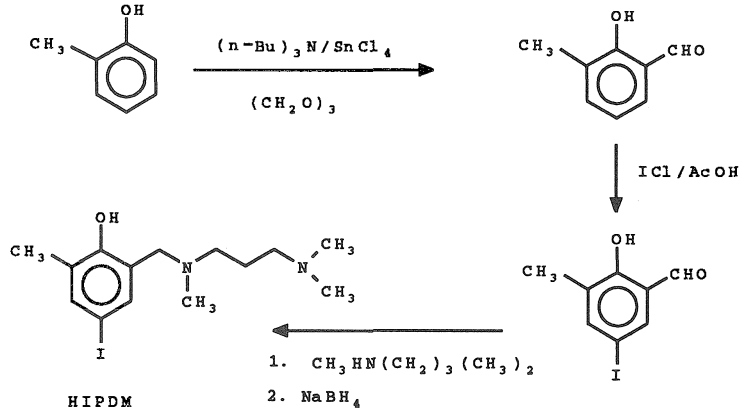
II. IMP AND HIPDM

A. Chemical Synthesis

The chemical synthesis of IMP and HIPDM are illustrated in Scheme 1 and 2. The iodo-aromatic ketones or aldehydes are condensed with an amine to give the corresponding imine, which is reduced by sodium borohydride to afford the desired product. The chemical reacations described in Schemes 1 and 2 are quite versatile, both of which have been employed to prepare a large number of IMP (25-27) and HIPDM analogs (28). It is interesting to note that IMP contains an asymmetric center (27); however, it appears that the brain uptake and retention of this compound is not affected by using either the S- or R-isomer (see discussion below).



Scheme 1. Chemical Synthesis of IMP



Scheme 2. Chemical Synthesis of HIPDM

B. Radiolabeling

Preparation of radioactive iodine labeled IMP or HIPDM is usually achieved by an iodine-iodine isotope exchange reaction. The exchange reaction for IMP requires more extensive heating (a melt condition) or the presence of a catalyst (either ammonium sulfate of Cu^{+}). Since the iodine atom of HIPDM is on an "activated" aromatic ring (by the presence of a

hydroxy group) the exchange reaction can be carried out in an aqueous acidic solution at a lower reaction temperature (100°C for 15-30 min). The latter procedure is amenable to kit formulation (28).

The activation energy for the HIPDM exchange reaction was determined to be 30.6 Kcal/mole (28), which is higher than that for IAP (23.3 Kcal/mole) (29). No similar evaluation has been reported for IMP, but it is likely that the value will be higher than that of HIPDM.

Other types of labeling reactions have been reported for IMP and HIPDM. [^{125}I]IMP can also be prepared by a Br-I exchange reaction (30). By separating the BrIMP from IMP on HPLC, carrier-free [^{125}I]IMP can be obtained. A second procedure using an organoborane precursor for preparing the carrier-free [^{125}I]IMP was also reported (31). Since the uptake and retention in the brain appears to be independent of the injected carrier at a wide dose range (see discussion below), the usefulness of such preparations is not apparent.

In order to prepare [^{122}I]HIPDM, (I-122,$t1_{/2}$ = 3.6 min) for PET rCBF imaging, an oxidative iodination procedure was developed (32). Using I-122 sodium iodide, chloramine and the uniodinated HIPDM starting material, the synthesis, purification and sterilization are completed in 3 minutes. The corresponding [^{122}I]IMP and its derivatives have also been prepared by employing a similar reaction procedure (33).

C. Biodistribution

Both IMP and HIPDM have been studied extensively in animals as a quantitative regional cerebral perfusion agent (34,35). Neither of these agents is perfect for blood flow measurement but, from a qualitative point of view, both have exhibited useful properties in various clinical situations. After an i.v. injection in humans, the agents localize in the brain rapidly, within 5 to 20

min (36,37). The delay in the arrival of the agent may be due to the initial uptake and washout from the capillaries of the lungs. HIPDM appears to have a faster brain uptake after i.v. injection. This net uptake is the result of a combination of influx and efflux from the brain tissue. There is disagreement over the exact amount of IMP and HIPDM taken up by the brain. It has been suggested that IMP displays a higher brain uptake than that of HIPDM (38). However, quantitative studies in humans showed that the total brain uptake for either one of the agents was generally 6-8% of the injected dose (39). The initial uptake sites other than brain are liver (30-60%) and lungs (40-50%) (37). IMP localizes more in liver than lungs while the reverse is true for HIPDM (6,10) (Fig. 4).

The brain retention is very long for both agents. For HIPDM the half life for brain retention was 40 hrs; a similar result was observed for IMP. The long retention time is of importance for SPECT imaging, based on a single head camera, which traditionally requires 30 to 60 min for adequate data collection. Another important factor to consider is the fixation of regional distribution. Due to the long data collection times used, it is essential that the agent remain fixed in the brain and little change in regional distribution occur while the data are being accumulated. Both IMP and HIPDM appear to be suitable with the time frame (30-60 min) needed for SPECT imaging.

Significant uptake of IMP in the eyes of dogs and monkeys has been observed; however, the same uptake has not been observed in humans (40). This difference in eye uptake may be related to the melanin content and/or other biogenic amine metabolic processes.

D. Clinical Application

For patients with various cerebral vascular abnormalities, cerebral perfusion imaging with IMP, HIPDM or Xe-133 can show the defect in

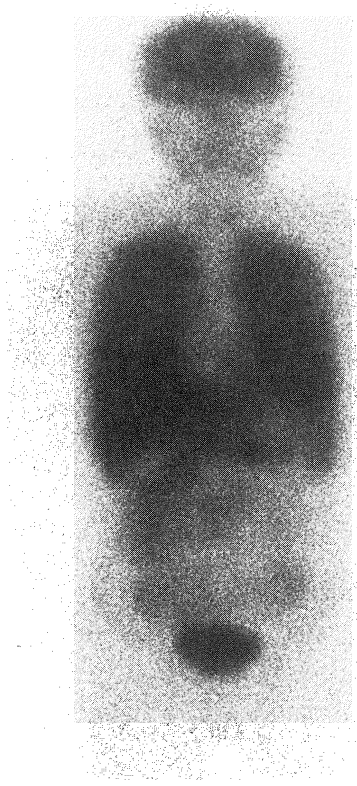


Figure 4. A Gamma Camera Image of a Patient Three Hours After I.V. Injection of [^{123}I]-HIPDM

regional blood supply (37,41-45). IMP or HIPDM perfusion images taken at a later time point (4 hr post injection) show reperfusion, which is filling in of the earlier deficit areas. This may be due to the presence of residual blood supply as well as the redistribution of the amines from liver or lungs to brain. A recent report (46) has suggested that acetazolamide, a carbonic anhydrase inhibitor producing temporary elevations in cerebral blood flow, may enhance the sensitivity for detection of cerebral vascular disease with HIPDM.

SPECT imaging in Alzheimer's patients showed a typical decrease of uptake in temporoparietal regions (44,48). This procedure may be of use for differential diagnosis of dementia patients suffering from Alzheimer's disease or other cerebral abnormalities such as multiple infarct dementia.

It is interesting to note that despite the profound decrease in perfusion to the temporoparietal cortex region, the distribution of muscarinic receptors, as measured by [^{123}I]QNB (3-quinuclidinyl-4-[^{123}I]iodobenzilate) in the corresponding region appeared to be preserved (48). The significance of this mismatch between the distribution of muscarinic receptors and the regional perfusion in the temporoparietal cortex remains unclear. Further study in the basic biochemistry and pathophysiology of Alzheimer's disease is needed before a widespread application of the diagnostic imaging technique can be achieved.

Several papers on rCBF evaluation of patients with epilepsy have been reported (49,50). In general, the brain images correlate well with EEG recordings. During the ictal (seizure) phase, focal increase in IMP and HIPDM uptake occurred in conjunction with behavioral and EEG evidence of seizure. While in the interictal phase, focally decreased rCBF occurred, at which time the EEG findings were negative or nonspecific.

There are conflicting reports on the uptake of IMP and HIPDM in brain tumors (51,52); both an increase and decrease in uptake of these agents in tumor regions has been observed. The inconsistent findings point to the fact that there are fundamental deficiencies in the understanding of tumor blood flow and transport, as well as the mechanism(s) of retention of these radioactive amines in normal and tumor tissue.

For [^{123}I]HIPDM, it has been reported that there is a significant uptake in the pancreas. SPECT imaging of the human pancreas has been reported (53,54), but the mechanism of this uptake is unclear. A recent report on using HIPDM to demonstrate pulmonary mass defects suggests that the agent may be

potentially useful for the detection of functional defects of the lung (55). The information may be complementary to that obtained with Tc-99m microspheres, in which the deficit of microsphere retention is based on pulmonary embolism.

E. Mechanisms of Uptake and Retention

The initial uptake of IMP and HIPDM in the brain is due to their high lipid solubility. The partition coefficient (1-octanol/pH 7.0 buffer) for IMP and HIPDM is 20 and 40, respectively. The first pass brain extraction is in the range of 85-95% (35,56), indicating that both agents can readily pass through the blood-brain barrier. The mechanism(s) of retention of IMP and HIPDM in brain is unclear. Several mechanisms have been suggested: pH-shift, specific or non-specific binding - however, none of these fully explains the prolonged retention in brain.

1. pH-SHIFT

Based on this hypothesis, it is suggested that the neutral and lipid-soluble radioactive amines can cross the blood-brain barrier by a simple diffusion mechanism. After the amines have entered the brain tissue, they are trapped due to the hydrogen ion concentration gradient (pH-shift) between blood and brain (7.4 vs 7.0, respectively) (21). The concentration gradient thus established should follow the physico-chemical principle based on the Henderson-Hasselbach equation (15,57). However, the concentration ratio between brain and blood is always higher than the predicted value, suggesting that a mechanism(s) other than pH-shift may also have an important contribution to the brain retention.

2. RECEPTOR BINDING

Since both IMP and HIPDM are amines with structures that can conceivably interact with neuronal or cellular receptors, it has been proposed that receptor binding may be the mechanism responsible for brain retention.

However, it has been demonstrated repeatedly that the brain uptake and retention of both agents is independent of carrier dose (24,58,59) (Fig. 5).

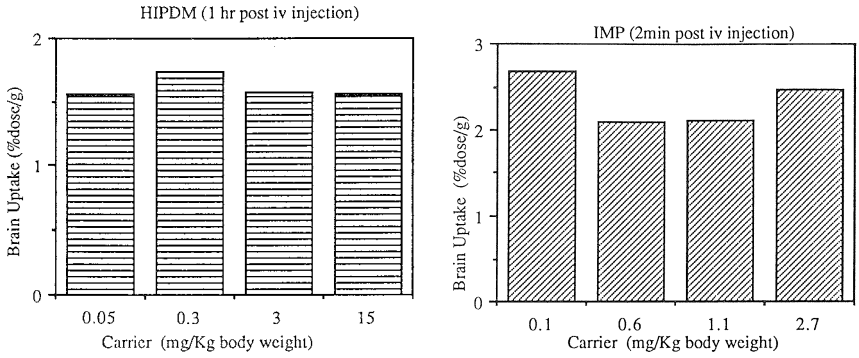


Figure 5. Carrier Effect on Brain Uptake of IMP and HIPDM in Rats

The basic criteria of a receptor specific agent are: (a) saturability and reversibility; and (b) physiological and pharmacological specificity (60,61). Neither of these criteria can be "remotely" related to the brain retention of IMP and HIPDM. In contrast to the expected saturability effect, higher carrier levels of the agents result in an increase in brain uptake (see discussion below). No measurable carrier effect on total brain uptake or regional cerebral distribution, by dissection or by in vivo autoradiography respectively, has been observed in animals, i.e. rats, mice, dogs, etc. No known pharmacological effect has been associated with these agents. Both optical isomers of IMP (R- and S-isomers) exhibit comparable brain uptake and retention (27). These data further suggest that the uptake and retention may not be related to receptor binding. It has been suggested that the binding is due to a "high capacity amine binding sites" in the brain. This hypothesis is inconsistent with the current definition of receptor binding. A preliminary report on the in vitro

[^{125}I]IMP autoradiograms of brain sections in the presence of various receptor agonists and antagonists, such as phenethylamine, glutamic acid and serotonin, displayed selective inhibition of uptake in various brain regions (62). This type of study is needed to provide further information on the nature of binding for IMP and HIPDM in various brain regions.

The lung uptake and retention of IMP and HIPDM appear to be related to a specific and saturable "binding" process (59, 63,64). At a higher carrier dose the lung uptake decreases (after an i.v. injection, the capillary bed in the lungs is the first tissue in contact with the injected dose); as a consequence, a higher amount of the injected dose is available for the brain uptake. Based on the saturation data published in the literature, the calculated total "binding capacity" for IMP and HIPDM in lung is about 1 umole/g of lung tissue. This number is at least three times higher than that of a "normal" receptor concentration. The saturation of the "binding" system may be related to a specific process of the endothelial cells in the lungs. Apparently, the process can also be blocked by pretreatment with imipramine and other related amines. Whether this is due to receptor site binding and/or metabolism in the lungs remains to be investigated.

Inhibition of norepinephrine and serotonin uptake and release in and from synaptosomes by IMP has been reported (20). However, the inhibition is generally effective at a concentration of 10^{-4}-10^{-6}M range. Considering the clinical dose of 1-2 mg of IMP and HIPDM in humans, the final concentration in the brain will be at the range of 10^{-9}M, which is three orders of magnitude lower than the effective concentration reported for the in vitro synapsomal uptake and release inhibition studies. It is possible that IMP and HIPDM may be taken up by mechanisms related to the presynaptic uptake of various biogenic amines. Further studies are needed to investigate the exact nature of such mechanisms in relation to the retention of these brain imaging agents.

III. OTHER IODINATED PERFUSION IMAGING AGENTS

A large number of iodinated amines as potential brain perfusion imaging agents have been reported in the literature, notably, [^{125}I]4-iodo-phentermine (IP) (65,66), phenylpiperazines and piperidines (Fig. 6).

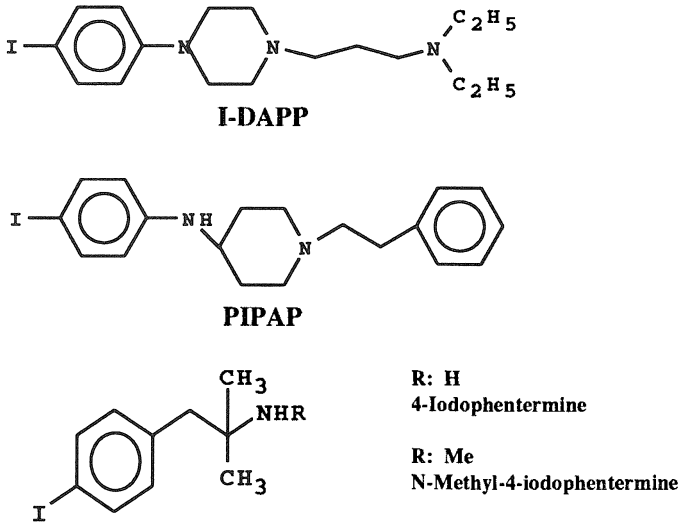


Figure 6. Other Iodinated Amines for Brain Perfusion Imaging

The design of the phentermines was based on their inability to serve as MAO or MFO enzyme substrate (due to the presence of dimethyl substitution on the carbon adjacent to the amine). These nonmetabolizable compounds show high brain uptake and retention; [^{125}I]IP shows very high initial uptake and essentially no washout from the brain (Fig. 7).

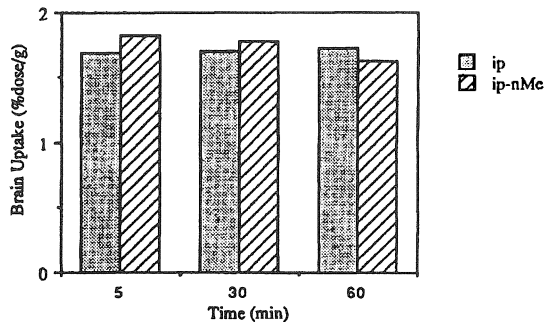


Figure 7. Brain Uptake of I-125 IP and C-11 IP-NMe in Rats

Similar high uptake and retention was reported for 4-[^{125}I]iodo-dimethylaminopropyl)-4-phenylpiperazine (IDAPP) (67) and 1-[2-phenylethyl]4-[N-[^{125}I]iodophenylamineo]-piperidine (PIPAP (68) (Fig. 8).

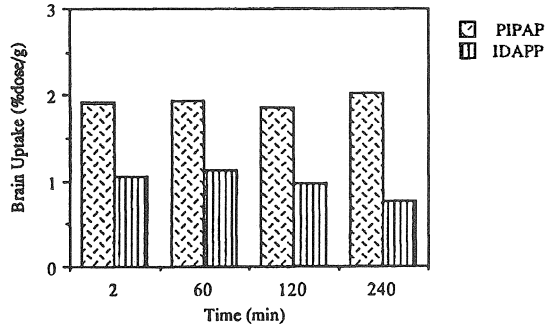


Figure 8. Brain Uptake of PIPAP and IDAPP in Rats

However, the mechanism of high uptake and persistent retention in brain for these seemingly diverse chemical structures has not been investigated. With [^{123}I]IMP and [^{123}I]HIPDM becoming available as rCBF agents and ^{99m}Tc-HMPAO as a potential replacement, there is little urgency for developing other iodinated perfusion imaging agents for the brain. Nonetheless, it is important to note that iodinated aromatic amines probably have a very different brain retention mechanism than that of ^{99m}Tc-HMPAO (see discussion in Chapter 2

by Dr. D. Nowotnik). Therefore, it cannot automatically be assumed that the iodinated aromatic amines and ^{99m}Tc-HMPAO are measuring the same physiological parameter(s).

Acknowledgements:

The author would like to thank Dr. Robert Mach, Ms. Jeanne Posthauer, and Mr. Jeffrey Billings for their assistance in preparing this manuscript. The work is partially supported by a grant awarded by the National Institutes of Health (NS-18509).

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The Development of Single-photon Emitting Receptor-binding Radiotracers

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I. INTRODUCTION: Flow Versus Receptor Binding

The greatest strength of nuclear medicine lies in its ability to detect and quantitate biologic function rather than fixed anatomical properties. These functional studies can be divided into two categories: the determination of flow and the determination of a biochemical reaction. These are not mutually exclusive phenomena; certainly, the material must be delivered to the target organ so flow is a necessary part of the distribution. Rather, the distinction is based on the relative kinetics of the two processes. Whether the distribution of a particular agent is a reflection of flow differences or of biochemical differences is kinetically determined. One example of a predominately flow-dominated distribution is radiolabeled microspheres where the trapping of the particles in the capillaries is much faster than the flow in comparable units (e.g. $time^{-1}$) so that flow is the rate determining step and hence the distribution is primarily a function of flow. In most cases, the relative kinetics are such that one process does not dominate the other kinetically over a wide range of flows.

Many of the radiopharmaceuticals used in routine clinical diagnosis

measure changes in flow to an organ as a function of disease. The gold standard for flow measurements in experimental animals is usually radiolabeled microspheres that are extracted by the capillary vessels with an extraction fraction of one. This extraction fraction is independent of flow. Many radiopharmaceuticals are compared to microspheres over a flow range expected in the clinic. For example, monocationic salts of the group I alkali metals, K, Rb and Cs, and the group III element Tl are indicators of flow and correlate with microspheres in a linear fashion over a limited range of flows (1). This is in spite of the fact that these cations are not strictly flow-limited tracers; that is, their extraction fraction is not one. Although the uptake of these cations is determined by capillary surface area and membrane permeability as well as flow, in practice the uptake is directly proportional to flow. However, this does not mean that the correlation between microspheres and a cation such as Rb will have a slope of one and an intercept of zero (2). The relative rate constants are such that the transport phenomena do not become kinetically dominant until the flow has reached multiples of the normal flow, but they do decrease the sensitivity to flow changes at lower flows (3). Other agents also measure flow. Certainly ^{99m}TcMAA, having an identical mechanism of localization as microspheres, is a measure of flow in the lung (4). Likewise, the extraction of colloid by the phagocytic cells in liver is flow limited (5). In the case of reduced flow as a result of hepatitis, the uptake of ^{99m}Tc sulfur colloid is decreased proportionately to flow (6). ^{131}I o-iodohippurate (Hippuran) is also used to measure flow, in this case, renal plasma flow because of its high extraction by the kidneys (7). McAfee et al, have shown that radiochemically pure Hippuran is extracted with high efficiency from plasma (8). Likewise, iodoamphetamine (9) and the pH shift agents of Kung et al. (10) are a measure of cerebral blood flow (11). Both seem to correlate well with microspheres immediately after injection in normal

tissue. But in abnormal tissue LeFrance et al. showed that the extraction decreased although the flow stayed the same (12). On the other hand, Szasz et al showed that increased concentration of radioactivity could be obtained in brain metastases with normal flow presumably due to increased amine uptake (13). Lucignani et al, showed that a related compound, HIPDM, was not a chemical microsphere, but rather a fortuitous balance of input and efflux led to constant levels of radioactivity (14). The extraction fraction for radiopharmaceuticals usually applies only to normal organs; no systematic study has identified the relative effect of decreased flow or changes in the physiological chemistry on decreased concentration of radioactivity in the diseased organ. The development of a flow tracer that is insensitive to biochemical changes, especially in disease states, has not been validated to date for single-photon emitting radiotracers.

In recent years the research emphasis has been toward not only those tracers that measure flow, but also those radiotracers that measure predominantly a biochemical process. This emphasis has come about for a number of reasons: the competition of other imaging modalities, the success in measuring biochemical pathways by using cycloton-produced radionuclides by isotopic substitution, and the improvements in both positron emission tomography and single-photon computed tomography. Many new imaging modalities such as CAT, nuclear magnetic resonance imaging, and ultrasound are better suited to record anatomical changes. On the other hand, the success of isotopic substitution with such compounds as (^{11}C)-palmitic acid (15), (^{11}C)-glucose (16), and (^{18}F)-2-fluoro-2-deoxyglucose (17) has demonstrated that in vivo biochemical tracers are possible. Finally, the demonstration that external imaging can be used to trace physiological chemistry in quantitative terms has had a major impact on the direction of research. The ability to measure glucose metabolism using (^{18}F)-2-fluoro-2-deoxyglucose has been especially impressive. In a similar manner, the ability to measure receptor

concentration by external detection would greatly broaden the field of biochemical radiotracers.

II. HISTORY OF RECEPTOR LIGANDS

A. Definition of a Receptor-Binding Ligand

There are several classes of biochemical radiotracers that can be studied. Of these classes, receptor binding radiotracers are especially interesting in that changes in receptor concentration are thought to be related to certain disease states. The receptor concept was developed to explain the specific effect of a minute amount of unchanged substrate on a target organ. Throughout the history of science, investigators realized that there must be a substance that imparts specificity to a particular cell type. Ehrlich realized this in his study of the interaction of antigens with specific, complementary, preformed receptors (18). In 1906, Langley postulated the existence of "receptive substances" on cell surfaces (19). Few receptors have been isolated for structural determination. As a result the definition of a receptor is operational (20). It is defined by certain properties observed in vitro. The usual criteria are high ligand affinity, specificity, saturability, and distribution in relation to physiologic response. These criteria separate the receptor from a binding site in that the latter is not involved in a physiologic action. An example of a such a binding site is thyroxine-binding globulin which binds thyroxine in plasma with high affinity and can be saturated but causes no physiologic action by virtue of the binding. The receptor can be differentiated from the enzyme in that the ligand causes a physiologic effect without molecular changes. The most important property of an enzyme is its catalytic activity which is influenced by the nature of the substrate, temperature and pH. The receptors have no catalytic activity, but rather produce a physiologic change indirectly. In addition, enzyme systems are often much higher capacity than receptor

systems.

The determination of the properties of receptors has been made possible by the development of high specific activity radiotracers. Jensen and Jacobson used radiolabeled estradiol to identify the cytosolic estradiol receptor in the early 1960s (21). In general, receptor protein is present in limited concentration, about 10^{-7} to 10^{-10}M in homogenized tissue. Therefore, the receptor is easily saturated by the appropriate ligand. Specificity and high ligand affinity are closely related because, by the nature of the high affinity between the receptor and the ligand, specificity results. Often ligands at high concentration can cause a physiologic effect at numerous receptors, but are specific for only one receptor at low concentration. In the central nervous system, specificity is imparted to relatively low affinity agonists by virtue of the synaptic connections which are insulated both morphologically and biochemically by diffusional barriers, high affinity neurotransmitter uptake systems, and strategically located catabolic enzymes (22). One of the most important properties of receptors is stereoselectivity in the case when a pair of stereoisomers exist, and one is far more potent than the other in binding affinity and physiologic response. For in vivo studies this property will allow proof of receptor binding because it can be tested using high specific activity, nonphysiologically-active radiotracers. In vivo tests for saturability would require the injection of a physiologically active amount of the biochemical or drug. This would probably be unacceptable as a routine diagnostic procedure if the ligand produces a pharmacologic effect. Many of the antagonist will produce adverse pharmacologic effects and some toxic reactions.

The final criterion is that the binding of the ligand to the sites can be related to the biological effect of the ligand. Experimentally, this has often been shown by comparing the affinity of various ligands with their in vivo biological effect. If a correlation is obtained, then the receptor is defined. In the radiotracer context, the distribution of the radioligand is determined in vivo

and this is correlated with the known distribution of receptors from biological studies in vivo or from in vitro assays of various organs and tissues.

In general, receptor binding radiotracers offer an area of research that will allow the noninvasive monitoring of the change in receptor as a function of disease. The development of receptor binding radiotracers is a two part problem: (1) the development of a radioligand that has a high receptor to nonreceptor binding and fulfills the operational definition and (2) the development of an analytical technique that shows a high sensitivity between the radioactivity in the target organ and the receptor concentration. Many radioligands have been shown to localize in receptor containing tissue and have fulfilled the operational definition for a receptor binding radiotracer. Few have fulfilled the second criteria.

B. Types of Receptor-Specific Radiopharmaceuticals

Katzenellenbogen et al. (23) in their review of the history of steroid receptor binding radiotracers quote Albert et al's 1949 work (24) as the earliest study in that field. This interest in radiolabeling steroid receptor ligands is reflected in the large number of compounds that soon appeared in the literature (23,25). Most were evaluated by in vivo distribution studies in small animals. The identification of cytosolic estradiol receptors in 1960 led to the systematic study of the requirements for receptor binding by those in the drug industry and those developing radiotracers (26). One of the first iodinated estrogens with a high affinity constant and high specific activity was 16-alpha iodoestradiol reported in 1979 (27). Of the iodinated steroids, 11-beta methoxy, 17-alpha iodovinyl estradiol is the best of the lot considering ease of synthesis, attainable effective and chemical specific activity, affinity constant, and nonspecific binding (28). The major focus of gamma-emitting receptor-binding radiotracers has definitely been on the steroid hormones (29). Ready

availability of a radionuclide was often the guiding force for the use of a radiolabel in the early studies. In this regard, iodine-131 has endured as one of the most popular of the gamma ray emitting radionuclides. The beta emitting radionuclide ^{128}I was the first iodine isotope studied in the life sciences, used only four years after the discovery of artificially produced radioactivity. ^{131}I and ^{125}I, which were discovered later, are today the most often used radionuclides for radiolabeling receptor-specific ligands along with ^{77}Br. Because the x-ray emissions of ^{125}I are of relatively low energy and cannot be detected easily by external imaging, this radionuclide is used primarily for in vitro or small animal work whereas ^{123}I, a cyclotron product, is used along with ^{131}I for in vivo imaging studies (30).

The first receptor system having the distinction of being studied using the highly desirable radionuclide ^{99m}Tc is the hepatic binding protein ^{99m}Tc galactosyl-neoglycoalbumin (NGA) (31,32,33). $^{99m}TcNGA$ is a radiolabeled ligand to hepatic binding protein (HBP), a receptor that is located at the plasma membrane of the hepatocytes. HBP normally recognizes and binds galactose-terminated glycoproteins that are transported to hepatic lysosomes where the ligand complex is catabolized and the receptor is subsequently recycled to the cell surface. NGA is formed by combining albumin with galactose in various molar ratios with subsequent ^{99m}Tc radiolabeling occurring via electrolysis. Because of the relatively low toxicity, this compound can be used in large enough concentrations so that the interaction between the ligand and the receptor are on the linear portion of the second order binding curve. This produces a second order response that is sensitive to the binding affinity, the ligand concentration, and the receptor concentration.

Stadalnik et al (34,35) studied ^{99m}Tc NGA in patients and found HBP concentration directly related to the clinical biochemical manifestations of reduced functional hepatocyte mass in liver disease, such as hepatoma, cirrhosis, and liver metastases.

C. Cerebral Receptor Binding Ligands

The neuroleptic receptor binding systems have received tremendous attention recently because of their importance in neurological disease and their potential for diagnosis. Friedman et al (36,37) studied ^{77}Br-bromo-spiroperidol in various species and concluded that this compound reached steady state rapidly compared to spiperone itself. They suggested that an equilibrium model could be used to account for specific binding and for competition of endogenous dopamine for the same D_2 receptors. They also found that ^{77}Br-bromospiperone was readily extracted and that the fraction of specific binding was high. While reasonably good images could be obtained using a pinhole collimator, the lack of availability and the poor imaging characteristics of ^{77}Br prevented extensive clinical studies. Br-77 bromo-benperidol is another substrate for D_2 receptors that achieves relatively high specific-to-non-specific ratio in primates (38,39). Even though this radiotracer binds to the receptor according to the operational definition of a receptor, the target to nontarget ratio is not as high as spiperone itself, perhaps due to the relatively high lipophilicity which increases nonspecific binding.

The muscarinic acetylcholine receptor (mAChR) system has also been studied extensively (40,41). The radioiodinated analog of 3-quinuclidinyl benzilate (QNB) has been shown to bind to the mAChR by testing the saturability and the stereoselectivity in the corpus striatum, cerebellum, and the heart of rats. 3-Quinuclidinyl 4-iodobenzilate (4-IQNB) receptor binding can be inhibited by co-injection of small amounts of nonradioactive mAChR ligands as well as displaced by the same materials after the 4-IQNB has bound to the receptor binding. The in vivo experiments involve a complicated set of variables including the total receptor concentration, the dissociation rate, transport of the displacing ligand, and the input function (4-IQNB still available for uptake from the blood). Nevertheless, the combined evidence

along with the regional distribution indicates a receptor-mediated localization. Another important proof is obtained by using two stereomers of IQNB differing in the chirality at the quinuclidinyl carbon. The difference in distribution, especially in those organs containing mAChR, between the pharmacologically active form, the 3-R-quinuclidinyl-4-iodobenzilate, and the pharmacologically inactive form, the 3-S-quinuclidinyl 4-iodobenzilate is striking. 4-IQNB was first studied in humans in May 1983, but since then only limited studies in disease states have been undertaken (42,43).

Many receptor systems have been studied and the necessary experiments by which to validate in vivo receptor binding are in place, but the transition from the validation of gamma-emitting, receptor-binding radiotracer to its use to determine the change in receptor concentration is a major step. Many have been validated, but few have been shown to be a sensitive probe of receptor concentration changes.

One of the most often studied receptor-binding radiotracers in man is the positron-emitting derivative ^{11}C-N-methylspiperone (CMS) (44) (see chapter by Langstrom). The dopamine D_2 and the serotonin S_2 receptors were imaged with ^{11}C-N-methylspiperone and measured in human volunteers. The relative receptor concentration was derived from tissue ratio data using the corpus striatum which contains D_2 receptors and the cerebellum which does not. Assuming that the change in tissue ratio is related to a change in receptor concentration, the D_2 and S_2 receptor concentration decreased as a function of age. Recently, the same group studied eleven normal volunteers, ten drug-naive and five previously treated schizophrenic patients (45). Although the tissue ratio using no carrier added CMS did not show a difference, the use of a high and low specific activity preparation resulted in the differentiation of the tissue ratio in normal and drug naive schizophrenics. As a result, the drug-

naive schizophrenics were thought to possess increased receptor concentration. Many receptor-binding radiotracers targeted for neurological receptors have been developed (46,47). Two major tasks remain before these radiopharmaceuticals become clinically useful. The ability to determine the receptor number and the sensitivity to change in receptor concentration must be validated. Then, using a radioligand deemed to be sensitive to receptor change, clinical studies must be carried out verifying their clinical sensitivity and selectivity. This is the challenge for both single-photon and positron emitting, receptor-binding radiotracers in the coming years.

III. CHOICE OF RADIONUCLIDE

A. Technetium

^{99m}Tc is by far the best radionuclide from an imaging point of view because of the ideal nuclear properties for the standard gamma camera and the ready availability afforded by the ^{99}Mo/^{99m}Tc generator system. However, the incorporation of ^{99m}Tc into a receptor antagonist is a formidable synthetic task which no doubt will perturb the interaction of the receptor ligand with the receptor. One important consideration is the preparation of a neutral chelate to better match the lipophilicity of most receptor binding ligands and to cross cell membranes in the case of cerebral receptors and peripheral intracellular receptors. Neutral ^{99m}Tc chelates have been prepared by Yokoyama et al with the KTS chelate (48), Burns et al with the diaminodithio ligands (49), Troutner et al with the amineoxime ligands (50) and Nunn et al. with the seven coordinate tris dioximes (51). Although the ^{99m}Tc chelates have been bound to various biochemicals, especially the fatty acids, none have been bound to receptor binding ligands in such a way as to maintain high receptor affinity. One receptor ligand that is labeled with ^{99m}Tc is neoglactosealbumin (NGA).

B. Halogens

Radiohalogens seem to be a more likely prospect for initial studies. Of the radiohalogens available for external imaging ^{123}I, ^{77}Br and ^{18}F are the most desirable based on a combination of nuclear chemical properties. Radioiodine has been one of the earliest and most often used radionuclides.

The radionuclides with the best nuclear properties for imaging with the gamma camera are ^{123}I and ^{131}I . ^{125}I has also been used but is most effective in in vitro tests.

Both ^{125}I and ^{131}I are reactor-produced and therefore less expensive and more readily available than the cyclotron product ^{123}I. However, the equilibrium absorbed dose to the patient from both reactor products is high because of their long half lives. ^{123}I is the ideal radioisotope for imaging with a 159 KeV gamma ray and low radiation absorbed dose. This gamma ray has a half thickness in water of 4.7 cm and therefore has satisfactory tissue penetration, yet the energy is low enough to be easily collimated (52). But this radionuclide is only produced in a cyclotron and cannot easily be made free of ^{124}I and/or ^{125}I with a low energy cyclotron. High energy protons, $^{127}I(p,5n)$, or high energy helium particles, $^{123}Te(a,3n)$, produce the purest ^{123}I but are not readily available (53). High purity ^{123}I is produced by those nuclear transformations that go through a ^{123}Xe intermediate. The most often used reaction to produce ^{123}I of high purity is the $^{127}I(p,5n)^{123}I$ reaction. The indirect method of production via intermediates does not result in ^{124}I because ^{124}Xe is stable. ^{124}I is an undesirable radionuclidic impurity because of its long half life (4.2 days) and its high energy photons (511, 603, 723 KeV). A 1% level of ^{124}I at the time of production will usually result in a 5% contamination at the imaging time of 24 h. The ^{124}I degrades the resolution because a significant fraction of the gamma rays within the 159 KeV energy window will be from scattered radiation. With a low energy collimator, the scatter contribution will be 28%. With a high resolution, medium-energy

collimator, the contribution is 10%. With a high energy collimator only 3% is scattered radiation, but the sensitivity is greatly reduced (54). Since high sensitivity is necessary for the external detection of receptor-binding radioligands, the need for high radionuclide purity is obvious.

^{123}I appears to be the best radiohalogen based on nuclear properties, absorbed radiation dose to the patient, and availability. However, the chemical properties of iodine are less than ideal because of the low bond energy of the carbon-iodine bond and the subsequent ease of in vivo deiodination. Bromine, on the other hand, has superior chemical properties to iodine in most instances but ^{77}Br has a complicated gamma ray decay scheme which makes image resolution difficult and the radiation dose to the patient higher per millicurie than that delivered by ^{123}I. A relative Figure of Merit for the halogens shows that $^{123}I > {}^{18}F >> {}^{77}Br$(55). Presently, hundred millicurie quantities of ^{77}Br are made only on large accelerators (56).

IV. CHOICE OF CHEMISTRY

A. Iodine

Based on the nuclear and chemical properties, it appears that ^{123}I and ^{99}mTc are the radionuclides of choice for radiolabeling receptor binding ligands. The chemical properties of iodine are a result of the decreasing ionization potential, larger atomic radii and the larger van der Waals forces and increased polarizability found as the atomic number increases in the group VII congeners.

Of the halogens, iodine is most likely to support a positive charge and thus is the least reactive toward electrophilic addition or substitution. Iodine also forms the weakest bonds to carbon and the other first row elements (60 kcal/mole for aromatic carbon-iodine bonds). Despite its anticipated stability, the I^+ ion does not exist alone, but usually forms a complex with a

nucleophilic species such as water or pyridine (57,58).

1. ELECTROPHILIC IODINATION OF ACTIVATED AROMATIC COMPOUNDS

In electrophilic substitution, the most reactive aromatic compound is phenol followed by aniline, methoxybenzene and imidazole. The anion seems to be the reactive species so that close attention must be paid to the pKa value and the pH of the reaction. Imidazole and pyrazole are iodinated in the 4 position and therefore fused ring compounds such as benzimidazole are not easily iodinated. Isooxazole, oxazole and thiazole are less reactive than imidazole. In other heterocyclic compounds, the order of iodination at a specific reaction site is 3-thiophene = 3-furan < 3-benzofuran = 2-benzothiophene <3-benzothiophene = 2-benzofuran << 2-thiophene << 2-furan. For benzothiophene and indole the 3-position is preferred although in the heterocyclic alone (thiophene and pyrrole) the 2-position is more reactive. The 2-position is most reactive in furan and benzofuran (59).

Pyridine is not easily iodinated at a carbon atom, but rather forms charge-transfer complexes with the halogen at the nitrogen. Aliphatic unsaturated compounds can be iodinated, but the carbon iodine bond is much less stable than the bond in aromatic compounds, although the mode of deiodination is different in each case. The most popular iodinating agents used to produce electrophilic substitution in activated aromatic rings are: 1) iodine (60); 2) iodine monochloride (61); 3) chloramine-T (62); 4) lactoperoxidase (63); 5) electrolysis (64); 6) the use of prelabeled ligands (65).

2. PRELABELED LIGANDS

Bolton and Hunter (65) have proposed iodinated 3-(4-hydroxy-phenyl) propionic acid N-hydroxysuccinimide ester as an indirect iodinating reagent which avoids many of the problems of direct iodination. In this indirect

method of iodination, the active ester is iodinated and purified from oxidizing and reducing agents before being mixed with the target molecule. In this way, the impurities in the iodide solution, the oxidizing agents such as chloramine-T and the reducing agents such as metabisulfate do not come into contact with the target molecule. The reaction results in the formation of an amide bond with the lysine groups of the target molecule. This allows the iodination of molecules which do not contain tyrosine or whose biological activity might be lowered by alteration at tyrosine rather than at the lysine moiety. The specific activity obtained is usually lower than that obtained with chloramine-T. The Bolton Hunter reagent is the most popular although other prelabeled ligands - iodoaniline which is converted to a reactive diazonium ion (66), methyl 3, 5 diiodo-p-hydroxybenzimidate (67), ([3-^{125}I]iodo-4-hydroxyphenyl) propionyl carbohydrazide (68), and N-chloroacetyl-[^{125}I] iodotyramine (69), have been offered as equally effective reagents. This indirect iodination has been used for some time in the preparation of iodinated tracers in radioimmunoassay. Iodinated-histamine,-tyramine, and -tyrosine methyl ester can be reacted with carboxymethyl groups to produce an iodinated derivative (70). However, the perturbation to receptor binding ligands is large and successful use of this approach has not been reported to date.

In the past, high specific activities were attained by using one or more iodine molecules per target molecule. Discouraged by the evidence that heavily iodinated species are present even at low molar ratios (71), that chlorocompounds might be present with chloramine-T, and encouraged by the development of HPLC, most investigators iodinate low molecular weight compounds at high target to iodide ratios and then separate the iodinated target molecule to attain maximum specific activity. Assuming one iodine per molecule and carrier free radioisotope, this maximum specific activity for ^{125}I is 2200 Ci/mmol, for ^{131}I, 16200 Ci/mmol, and for ^{123}I 236,000 Ci/mmol.

This specific activity is not reached for ^{131}I prepared from natural tellurium because ^{127}I and ^{129}I are also produced (72). Depending on the irradiation time and the time after irradiation, different specific activities can be obtained (73). Higher specific activity ^{131}I can be obtained by uranium fission. Using enriched ^{130}Te, maximum specific activity ^{131}I can be produced, and this is now the method of choice.

3. ELECTROPHILIC IODINATION OF NONACTIVATED AROMATIC COMPOUNDS

A number of reagents have been suggested to iodinate benzene or deactivated aromatic rings. Most of these require strong acid to facilitate the reaction: HNO_3 (74), HIO_4 (75), peracetic acid (76), H_2SO_4 (77), and Ag_2SO_4 in H_2SO_4 (78). Gillespie and Morton (79) have reviewed the evidence for iodine cations. I_3^+ and I_5^+ cations have been found in concentrated H_2SO_4 and fluorosulphuric acid. The I_2^+ and I_4^+ cations were detected in solutions of fluorosulphuric acid oxidized with peroxydisulphuryl difluoride. Most of these acid solutions have not been used to radiolabel biomolecules. For instance Derbyshire's method of iodination caused oxidation of quinuclidinyl benzilate to benzophenone (80). Likewise other molecules with substituents sensitive to strong acids will not yield radiochemically pure products or high yields using these reagents.

Fortunately, electrophilic iodination can be carried out with reagents that react in dilute acid or in organic solvents. Some of the more popular reagents are: F_3CCO_2Ag/I_2 (81), $(CH_3CO_2)_2Hg/I_2$ (82). $TI(CF_3CO_2)_2/KI$ (83), $CuCl_2/I_2$ (84) electrolysis (85), H_2O_2/I_2 (86), ICl (87) and various chloroamines first investigated by Orton (88). However, those methods that require I_2 will not produce the required high specific activity for receptor-

binding radiotracers.

4. IODINATION OF AROMATIC RINGS BY REPLACEMENT OF OTHER SUBSTITUENTS

A large number of metal derivatives have been prepared to lend specificity and reactivity to iodination of deactivated rings. Various leaving groups have been studied both at the carrier iodine level and the carrier free level. Iododeboronation (89), iododesilylation (90), and iododestannylation (91) are among the more popular for carrier free reactions. These methods were recently compared in an attempt to incorporate short lived ^{122}I (T1/2 = 3.6 min) into aromatic rings (92). Because of the short-half life of ^{122}I, the relative reactivities were important. Likewise, it will be important to radiolabel the receptor binding radiotracers by efficient methods with ^{123}I so that the procedures can become a clinical reality. Of the five metal groups substituted on benzene, $SnMe_3$ and $HgCl_2$ were clearly the most reactive either in ethanol or acetic acid solvent. The oxidizing agent used to carry out the electrophilic substitution was dichloramine-T(DCT). The regioselectivity of these reagents is important for receptor binding radiotracers because of the structure-distribution sensitivity. It also permits the facile iodination of nonactivated aromatic compounds and in fact the aromatic compound must be deactivated to prevent competitive deprotonation reactions.

5. NUCLEOPHILIC IODINATION

Nucleophilic iodination of aromatic rings is usually carried out using either a diazonium ion prepared *in situ* or a triazene derivative. The mechanism of decomposition of these derivatives has been discussed at great length in the literature (93,94) The reaction mechanism rivals electrophilic iodination in terms of complexity and no simple theory has emerged. Szele and Zollinger (95) have presented an explanation for the effect of solvents on

competitive heterolytic and homolytic dediazoniation. In most of the earlier studies in acidic aqueous solution heterolytic dissociation to give an aryl cation and molecular nitrogen was the mechanism of choice. However, in other solvents such as 2,2,2-trifluoroethanol (TFE) at least two intermediates must be involved: the phenyl cation and a tight ion-pair between the phenyl cation and nitrogen. Solvent effects on heterolytic dediazoniation are small; the difference between the slowest and fastest is only a factor of 10. Radical reactions are also prominent with abstraction of a hydrogen atom to give the original aryl compound. The radical reaction is more dependent on the solvent. The formation of a radical pair and N_2 is favored by the nucleophilicity of the anion present. But the anion must be a good homolytic leaving group. Szele and Zollinger call this "the concept of a nucleofugic homolytic leaving group."

6. IODINE REPLACEMENT BY IODINE (IPSO REACTION)

One of the most popular methods of introducing radioiodine into aromatic structures is by halogen exchange. Stocklin (96) has outlined three methods: the melt method, low pH halogen exchange reactions, and excitation labeling. These methods do not, in general, produce radioligands with specific activities high enough to be used for receptor-binding radiotracers.

7. BROMINE REPLACEMENT BY IODINE

Stocklin in his review (96) also mentions Br for I exchange analogous to the ipso reaction. Recently, Wieland's group have adapted the original ammonium sulfate method (97) to produce higher specific activity ligands starting with the bromoderivative (98).

B. Technetium

Of the conveniently available radionuclides, technetium has by far the best

nuclear properties for diagnostic imaging. With the advent of commercial generator systems, instant technetium, innovations in chelation, and new chelating agents, there has been a marked expansion in the use of ^{99m}Tc labeled compounds. Chemical forms of ^{99m}Tc are presently the most widely used radiopharmaceuticals for radionuclide imaging of the brain, liver, lung, and skeleton, and to a lesser extent in thyroid scintigraphy. Compounds for both static and dynamic evaluation of renal and cardiac pathology have gained popularity with increasing computer technology.

In general, the use of these radiopharmaceuticals is based on the ability of specific organs to remove foreign substances from the blood. The ^{99m}Tc chelates for kidney studies are low molecular weight, water soluble compounds which are rapidly excreted via the renal pathway, hence, images of the kidneys are obtained. For hepatic imaging the most common ^{99m}Tc agent is radiolabeled colloid which is visualized following rapid phagocytosis by the reticuloendothelial system of the liver. The basis for pulmonary artery blood flow evaluation is the temporary mechanical obstruction of an innocuous percentage of the arteriolar-capillary pulmonary circulation by technetium-labeled particles ranging in size from 10 to 50 um.

For future progress in nuclear medicine, however, it appears that a more refined, specific approach to compound localization, which depends upon the use of radiolabeled biologically active compounds or synthetic drugs, will be needed. In spite of the potential rewards offered by this type of investigation, glaring difficulties have become evident in attempts to directly radiolabel the functional groups of compounds such as receptor-binding ligands. Firstly, the native functional group(s) may be needed to interact with the active biological site responsible for compound localization; should the radiolabel interfere with this, the normal behavior of the molecule will be altered and tracer studies will be a failure. Secondly, the radionuclide may bind to the molecule with

insufficient affinity to produce a stable chelate. Just as with the previous problem, the desired behavior of the labeled compound will not be achieved.

The importance of both of these factors is exemplified by radiolabeled bleomycin. Although it is not a receptor-binding radioligand, it is an ideal example of the chelate chemistry involved. Bleomycin is a mixture of closely related antibiotics that have been used successfully to treat a variety of malignancies. This antibiotic is a chelating agent and has been shown to bind a number of divalent and cations, but with varing affinities (99). Although chelates of indium, gallium and copper have not demonstrated the necessary in vivo and in vitro stability, the bond in cobalt bleomycin is more stable (100). Ideally, a technetium labeled bleomycin would be the most efficacious form; however, inspection of the bleomycin structure (101) indicates that the disaccharide moiety is the most likely chelating group but unfortunately, this is a low affinity site for ^{99m}Tc. This statement is based on work by Richards and Steigman (102) who demonstrated that the sugar moiety has a high affinity for ^{99m}Tc at pH 10-12, but a weak affinity at neutral pH. Therefore, the use of the native functional groups of bleomycin to bind a tracer such as technetium, results in a weak chelate with poor stability

Another factor for consideration is the change in biological activity of a drug or biological derivative secondary to the addition of a radiolabel. It has been shown that the chelation of copper to bleomycin destroys its ability to cleave strands of DNA (103). When labeled with cobalt, the antibacterial activity of Bleomycin is deleteriously affected and becomes negligible when tested against the usually responsive Bacillus subtilis ATC 6633 (104). In this instance, the cobalt appears to alter the biological effectiveness of the bleomycin because of its bond to the functional groups responsible for maintaining the antibiotic integrity of this drug.

Most of the attempts to label biochemicals have centered on fatty acid derivatives (105-114). None of the ^{99m}Tc labeled fatty acid derivatives have

distributed in vivo in the expected manner based on ^{11}C palmitic acid. Fatty acid derivatives containing a neutral chelate did not produce an improvement over those containing a charged chelate. At present, no ^{99m}Tc labeled receptor-binding radiotracers of MW $\leq$1000 have been developed. As mentioned earlier, the single example of a ^{99m}Tc labeled receptor-binding radiotracer is ^{99m}Tc NGA.

C. Review of Receptor-Binding Ligand Chemistry

Because of the need for high specific activity, the major method combines no-carrier-added iodide with an oxidizing agent such as Chloramine-T (Section IV, A-1). The recent attempts to produce iodinated receptor-binding radiotracers are outlined in Table 1. The method of iodination is given by reference to Section IV-A, Iodination Procedures 1 to 7.

Table 1. Iodinated Receptor-Binding Radiotracers

Ligand	Receptor System	Method	Ref.
1. LSD	5-HT_2	A-1	115
2. Iodopinodolol	beta adrenoceptor	A-1	116
3. IQNB	mAChR	A-5	117
4. Iodovinyl estradiol	estrogen	A-4	118
5. 11β-methoxy-17α-iodovinylestradiol	estrogen	A-4	119
6. Iodohydroxyphenylisopropyladenosine	adenosine	A-1	120
7. 2-[β-iodohydroxyphenyl-ethyl]aminomethyl tetralone	alpha	A-1	121
8. 16α-iodoestradiol	estrogen	A-7	122
9. Iodohydroxyphenylthromboxane der.	Thromboxane A_2 Prostaglandin H_2	A-1	123
10. 4-iodospiroperidol	Dopamine D2	A-1	124
11. iododilactitol tyramine	LDL	A-2	125
12. 6,7-dimethoxy-4-(4-amino-3-iodobenzyl)isoquinoline	Isoquinoline	A-1	126
13. 7-amino-8-iodoketanserin	Serotonin S_2	A-1	127
14. 6-iodo-androsten-5-enes	Androgen	A-4	128

Table 1 (cont'd.) Ligand	Receptor System	Method	Ref.
15. 6-iodo-pregnen-5-enes	Progesterone	A-4	128
16. 17α-iodovinylnortestosterone	Androgen	A-4	129
17. 2-iodospiperone	Dopamine D_2		130
18. Iodobenzyl ZM (IBZM)	Dopamine D_2	A-1	131
19. Iodo SCH 23982	Dopamine D_1	A-1	132
20. 15α,11β-methoxyestradiol	Estrogen	A-4	133
21. iodoraclopride	Dopamine D_2	A-1	131
22. IodoRo5-4864	Peripheral Benzodiazepine	A-6	134
23. Iodo-flunitrazepam	Peripheral Benzodiazepine	A-5	135
24. Iodo-PK 11195	Peripheral Benzodiazepine	A-7	136

V. STRUCTURE-DISTRIBUTION RELATIONSHIPS

Structure-activity relationships have been an important part of pharmaceutical development. Because of the propensity toward orally-absorbed drugs, pharmaceutical research has been faced with the problems of cell membrane transport. Radiopharmaceuticals, on the other hand, are mostly injected intravenously, and as a result there have been fewer studies concerned with structure-distribution relationships. With the advent of radioligands that cross normal cell membranes, this has become an important part of the investigational studies. With receptor binding radiotracers, another factor, receptor binding affinity, enters into the study. A great number of investigations, mostly carried out in vitro, have been used to identify high affinity antagonist. The combination of the two structure-distribution relationships (SDRs) has now become an important part of the development of receptor-binding radiotracers.

A. Physicochemical Properties

Most radiopharmaceuticals to date are water-soluble, polar compounds that are excreted by the kidneys. As radioligands were developed that cross cell membranes, the properties of these compounds were often correlated with a particular physicochemical property. Most often the property is lipophilicity determined either by measuring the organic-aqueous partition coefficient or using a related technique such as reversed phase high pressure liquid chromatography. Various organic solvents have been used to determine the partition coefficient. The goal is to choose an organic solvent that most closely models the cell membrane. Franks and Lieb published the classic example of the relationship between the activity of general anesthetics and the partition coefficient (137). Various solvents were used including vegetable oil, n-hexadecane and n-octanol. The vegetable oil system did not give a good correlation when hydroxy-containing anesthetics were included, and the hexadecane system was poor for most polar anesthetics. Octanol-water on the other hand, produced a good correlation for a broad range of anesthetics. The authors concluded that octanol best represents the cell membrane involved in anesthesia.

Oftentimes, a poor correlation is not due to the incorrect choice of solvent but rather to the dependence on a diffusion phenomena. The net flux across a cell membrane is directly proportional to the diffusion coefficient and the partition coefficient of the ligand and indirectly proportional to the thickness of the membrane. The combination of these three parameters is the permeability coefficient. If diffusion is an important parameter in the cell membrane transport, then it must be taken into account. Derivations of the size-corrected permeability coefficient then become an important part of the evaluation. These can be calculated by measuring the permeability coefficient-partition coefficient ratio as a function of the molecular volume. Since the molecular

volume is difficult to obtain for anything but the simplest organic compounds, a function of the molecular weight (MW), usually the square root, is often used (138).

The protocol for choosing the correct partitioning model (139) involves separating the partitioning step from the diffusion step.

1. OBTAIN THE PARTITION COEFFICIENT IN VARIOUS SOLVENT-WATER SYSTEMS

a. Estimate the dependence of the diffusion coefficient on the van der Waals volume and calculate the volume-independent diffusion coefficient. This can be obtained by plotting the permeability coefficient divided by the partition coefficient (which is a function of the diffusion coefficient) versus the molecular volume, or a function thereof.

b. Plot the size-corrected permeability coefficient against the partition coefficient for various solvents to determine which is the best linear model.

2. DETERMINE THE SIZE-CORRECTED PERMEABILITY COEFFICIENT

In the radiopharmaceutical context the % dose/g tissue gives a value proportional to the permeability coefficient. The dependence on molecular weight can be determined and then the molecular weight independent % dose/g can be plotted against the partition coefficient to determine the best linear model. This is more sophisticated than the model by Fenstermacher et al. (140) because it determines the molecular volume dependence for each membrane. It is more difficult because the molecule volume must be estimated. Recently, Wilson and Pinkerton (141) used various calibrated chromatographic techniques to estimate the volume of ^{99m}Tc phosphonates.

SDRs for various radiopharmaceuticals have been published over the years. Burns et al correlated % biliary excretion of various HIDA analogs with

the ln MW/Z where Z is the charge on the ^{99m}Tc radiopharmaceutical (142). Nunn et al related the calculated lipophilicity and the reversed phase HPLC retention time to the % urinary excretion of some 33 HIDA derivatives labeled with ^{99m}Tc (143). The measured and calculated lipophilicities were correlated, but the ortho substituents and those without ortho substituents gave different slopes and intercepts. However, the measured lipophilicity of ^{99m}Tc HIDAs from both groups correlated well with the % renal excretion.

Other SDRs have been published, but the emphasis has been on structural changes in the molecule without concomitant measurements of physical chemical properties. Wieland et al have described various substituted aralkyl-guanidines and the effect of these substituents on the adrenal medulla uptake of radioactivity (144). Likewise, Counsell et al studied the effect of substituents on the distribution of radiolabeled androgens (145). Spitznagle et al followed a similar approach for derivatives of cortisol (146). A number of these and other SDRs have been published as a Symposium Proceedings (147).

B. Receptor Binding Tests

For receptor-binding radiotracers, especially those for cerebral receptors, the SDR can be subdivided into two aspects: transport and receptor binding. For the cerebral agents, there have been three SDR studies to date. The % dose/g in the rat brain was correlated with the octanol-water partition coefficient for a series of dopamine receptor binding ligands by Moerlein et al (148). For the various spiperone analogues, the molecular weight varied by only ca. 30% but the octanol-water partition coefficient varied by 2.5 log units from 2.7 to 5.2. The striatum to cerebellum ratio is an indication of the receptor binding since the striatum contains D_2 dopamine receptors and the cerebellum does not. This ratio is at a maximum for N-methylspiperone at log P = 3.2, but the striatum concentration peaks for bromospiperone with a log P

of 3.6. In this case, the log P value is important for both delivery to the brain and to obtaining a specific receptor binding ratio. Fortunately, the in vitro binding constant for the series of 6 pharmaceuticals varied from only 2.6 to 10.3 nM so that these were essentially one variable experiments.

Welch et al (149) looked at similar spiperone derivatives that were labeled with ^{18}F rather than ^{77}Br as in the case of Moerlein et al (150). Welch et al did not find the same dependence in the % dose/brain or in the striatum to cerebellum ratio although the striatum to cerebellum ratio is highest for the N-methyl derivative.

Finally, Rzeszotarski et al applied the NIH-EPA Chemical Information System to determine various physical-chemical parameters of derivatives of 3-quinuclidinyl benzilate, a strong antagonist toward the muscarinic cholinergic receptor (151). These parameters are shown in Table 2.

The preliminary results permit the following conclusions: a) 3-Quinuclidinol provides the most compact, rigid aminoalcohol moiety with the greatest accessibility of the lone electron pair on the nitrogen atom and therefore any search for a better aminoalcohol moiety will be difficult. b) The replacement of one of the phenyl rings of QNB with a cyclopentyl group leads to substantial improvement of affinity in the heart and displaceability in the brain. When the benzylic acid part is replaced by xanthene-9-carboxylic acid, a significant difference in affinity to the receptor in the two tissues is observed. In these cases the shape, volume and lipophilicity of the molecules play the determining role.

The logit plot of the displacement of ^{3}H-QNB by each of the analogues is

Table 2. Physicochemical and Biochemical Properties of QNB Derivatives

	K_A[a]		k'[b]	Calc. Log P	Surface Area (A^2)		Mol. Vol. (A^3)	FH_2O[c] (Kcal/mol
	RVM	RC			Unsolv.	Solv.		
QNB	5.28	---	1.6	2.87	136.13	315.56	169.10	-0.38
C_5H_9	8.21	3.58	2.5/2.9	3.05	146.69	318.02	211.10	1.36
C_4H_9	2.91	3.44	2.1/2.4	2.99	144.20	324.24	192.30	2.20
$HC{=}CCH_2$	0.03	1.26	1.3	2.17	98.29	269.71	115.00	0.63
QXN	0.225	3.56	1.5	2.74	225.56	439.46	314.70	-8.43
$3\text{-}FC_6H_4$	5.03	3.91	2.3	3.10	140.96	322.60	172.20	-4.54
$4\text{-}FC_6H_4$	3.03	3.97	2.3	3.03	140.96	322.60	172.40	-4.54
$2\text{-}BrC_6H_4$	0.598	0.66	3.3	3.71	155.39	344.11	192.00	--
$3\text{-}BrC_6H_4$	0.433	2.2	3.5	3.83	162.49	355.93	192.00	--
$4\text{-}BrC_6H_4$	0.608	1.81	4.3	4.06	162.49	355.93	192.00	--
$3\text{-}IC_6H_4$	0.482	1.52	4.0	4.05	168.19	365.22	202.50	--
$4\text{-}IC_6H_4$	1.22	2.37	5.0	4.30	168.19	365.22	202.50	--

(a) Association constant ($K_A \times 10^{-9}M^{-1}$) determined in vitro using either rat ventricular muscle (RVM) or rabbit caudate (RC) as the source of muscarinic cholinergic receptor.

(b) k' from reversed phase C_{18}HPLC with 5 mM octane sulfonic acid, pH 4, CH_3OH:THF:H_2O (26:16:58) as eluent.

(c) Free energy of solvation.

shown in Fig. 1 (152). The Y axis is the % dose/g bound in the brain in the presence of 50 nmol of nonradioactive analogue per animal to the percent dose/g bound using ^{3}H-QNB alone. The X axis is the relative binding as measured in the in vitro radioreceptor assay. The single compound that is less potent than it should be is 3-quinuclidinyl-4-iodobenzilate.

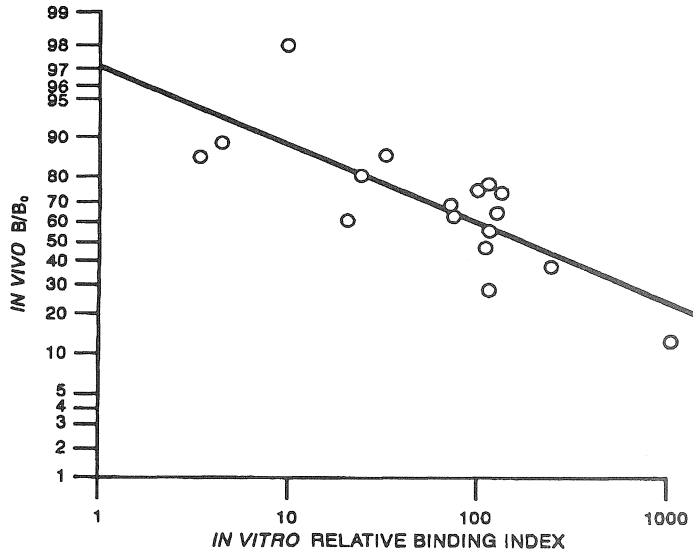

Figure 1. In-Vitro Relative Binding Index

From the studies of Moerlein et al and Welch et al, it is clear that transport into the brain is a function of the lipophilicity of the compound. For compounds of near equal affinity, this is the important factor in cerebral concentration. If, however, the affinity constants vary, then the amount in the cerebrum can be correlated with the affinity constant as shown for the QNB series. Katzenellenbogen has shown a similar dependence on affinity constant for a series of estrogens (153). Using the affinity constant alone was not, however, as highly correlated with the uterine concentration as when the affinity constant was adjusted by the lipophilicity of the estrogen. This

approach combines the permeability and receptor binding in a single index. Great strides have been made, nevertheless, in choosing receptor binding radiotracers by using the affinity constant alone, determined in vitro, to predict the target to nontarget ratio using the equilibrium expression developed by Scatchard (154).

$$\text{Bound/Free} = \text{Target to Non-target} = RoK\text{-}BK$$

Where Ro is the total receptor concentration,
K is the affinity constant and
B is the bound concentration.

At small B/Ro ratios, the target to nontarget ratio can be predicted by the product of the receptor concentration and the affinity constant, RoK.

VI. DETERMINATION OF RECEPTOR CONCENTRATION BY EXTERNAL IMAGING

Although many ligands have been labeled with radioiodine and neoglactosealbumin has been labeled with Tc, the in vivo data to date have been used mostly to support the contention that the radioactivity is receptor bound in the target organ. As stated earlier, the development of receptor binding radiotracer is a two-part problem: (1) the development of a radioligand that has a high receptor to nonreceptor binding and fulfills the operational definition and (2) the development of an analytical technique that shows a high sensitivity between the radioactivity in the target organ and the receptor concentration. Many experiments have been put forth to support the former, but few have been put forth to support the latter. The kinetic analysis has involved two approaches: (1) the use of a high specific activity radioligand and (2) the use of a low specific activity ligand or the ligand at varying specific activities.

A. Low Specific Activity Ligands

In a conference on receptor binding radiotracers in 1981, Krohn et al put forth the hypothesis that the maximum sensitivity to receptor concentration change will come at ligand to receptor ratios of between 0.2 to 0.8 (155). The ability to separate the total receptor concentration from the rate of ligand-receptor binding was also related to the receptor saturation level but, in most cases, investigators have treated the product of the receptor concentration and the binding rate constant as a single variable. The assumption that the binding rate constant has not changed in disease has addressed the latter issue but the former issue is still a major point of discussion. The sensitivity of determining receptor concentration change is best studied using simulations since there are few animal models where the receptor concentration can be systematically changed. An elegant analysis have been carried out by Vera et al that shows the coefficient of variation in the measurement of receptor concentration as a function of the binding affinity and the fractional receptor saturation. The receptor concentration is more precisely determined at higher fractional receptor saturation (156). The precision of determining receptor concentration has a parabolic dependence on the binding rate constant. At low ligand-receptor binding rate constant, the fraction of receptor binding to nonreceptor binding is low. At a very high rate constant, the radioactivity determined in the target organ is independent of receptor concentration and the rate determining step becomes the flow or membrane transport rather than the ligand receptor binding process.

Friedman et al used thermodynamic equilibrium to describe the binding process (157). This approach assumes that steady state is reached and that the equilibrium equations apply. Based on these equations, a number of simulations were run showing the effect of injected dose (specific activity) and endogenous ligand. These show the same results put forth by Vera et al, namely that the sensitivity to receptor change is maximal at about 50%

saturation. Farde et al have used a similar approach with ^{11}C raclopride (158).

Farde et al are using the law of mass action in the form of the Hill equation. This is the same equilibrium expression put forth by Katzenellenbogen et al (159) and Eckelman et al (160) to predict in vivo behavior using in vitro data. In general, the equation could predict trends but did not give the quantitative target to nontarget ratio. Therefore, the assumption listed by Farde et al to obtain an accurate receptor concentration in vivo are crucial. Three (3) of their assumptions involve the use of the caudate putamen to represent receptor binding and the use of the cerebellum to represent free ligand available for uptake. Then using four (4) different chemical doses of ^{11}C raclopride, a Hill plot can be constructed. Another key assumption is the requirement for equilibrium (or more properly steady state). If a process such as blood clearance or lung clearance is fast compared to the dissociation rate of the ligand-receptor complex then, the target to nontarget ratio will be decreased as shown in the equation derived by John Wagner

$$\frac{B}{F} = \frac{k_{12}}{k_{21}\text{-beta}}$$

where k_{12} is the rate of ligand-receptor association,
k_{21} is the rate of ligand-receptor dissociation and
beta is the rate of disappearance of the input function (161).

These approximations may result in a relative receptor value but exact correlation with in vitro values are most likely coincidental. Also the use of low specific activity ligand may cause physiologic effects that will alter the pharmacodynamics of the ligand. Welch et al showed some years ago that haloperidol membrane transport was dependent on the drug concentration.

B. High Specific Activity Ligands

The use of high specific activity ligands to estimate the receptor concentrations was first developed on an empirical basis using ^{3}H ligands. Wagner et al used the same approach and found that the slope of the tissue ratio versus time plot changes as a function of age. They used the D_2 receptor binding ligand ^{11}C-N-methylspiperone (CMS) and thus postulated that the D_2 receptor is decreasing as a function of age The analysis can be derived from basic principles as has been done by Patlak for an irreversible binding ligand and then applied to the three (3) compartment model. In this situation, the slope of the tissue ratio versus time plot represents a combination of rate constants:

$$\text{Slope} = \frac{k_2 k_3}{k_2 + k_3}$$

Where k_2 is the efflux from the extracellular fluid to the plasma

and k_3 is the first order process describing the ligand-receptor interaction.

K_3 is a pseudo-first order rate constant derived from the product of the rate constant (M^{-1}) and the receptor concentration (M^{-1}). For high specific activity ligands the free receptor concentration equals the total receptor concentration if the low affinity endogenous ligands are ignored. If k_2 is greater than k_3, then the slope will be a function of k_3 and hence the receptor concentration. But if $k_3 > k_2$, then the slope will be a function of membrane transport or blood flow. These constants have not been determined for ^{11}C-CMS.

Sawada et al. have recently analyzed 3-quinuclidinyl 4-iodobenzilate

(IQNB) using numerous kinetic models (162). They used a combination of low error and accuracy (using in vitro data as the gold standard) to choose the best model. They also applied the Patlak equation to IQNB for the three (3) compartment model (163). In the rat, the k_3/k_2 ratio was 1.4 showing that the slope of the tissue ratio versus time plot will be sensitive to changes in receptor concentration. From these values, one could expect that the tissue ratio (in the case of IQNB the caudate-putamen to cerebellum ratio) versus time plot would yield an apparent receptor concentration decrease of 70% when the receptor concentration actually decreased to 50% of the normal value in the rat.

Mintun et al. (164) have argued against the tissue ratio approach put forth by Wagner et al (165) because it lumps various physiologic process by using a single three (3) compartment model. Much controversy has resulted but few definitive experiments have been carried out to show the responsiveness of any parameters to a change in receptor concentration. The lack of a reliable animal model where well-validated receptor concentration changes take place has been a major impediment.

VII. CONCLUSION

Receptor binding radiotracers offer great promise in that they will lead to the noninvasive measurement of the change in receptor concentration as a function of disease. The single-photon emitting radiotracers, labeled with ^{123}I or ^{99m}Tc, are compromised by the instrumentation available especially for neuroreceptor studies and by the lack of routine availability of ^{123}I.

Instrumentation for single-photon emitting radiotracers has not progressed to the extent as that for position emitting radiotracers. However, multislice ring machine for single-photon emitting radiotracers are now available: the Triad (166), the Prism (167), the Strichman machine (168), the MUMPI (169),

and the ASPECT (170). The lack of readily available ring machines is most detrimental in cerebral receptor studies where the structures are small and the % dose/g is also small, but also applies to cardiac receptors and receptor dependent tumors.

The lack of availability of ^{123}I is also a major problem. The success of nuclear medicine has depended on the ready availability of ^{99m}Tc through the generator system. The positron-emitting radiotracers (^{11}C, ^{18}F) can also be produced by an on-site generator, a cyclotron. Although much more expensive than the ^{99m}Tc generator, it does allow for easy access. However, there is no generator system for ^{123}I and it cannot be made at high purity on the biomedical cyclotrons. A recent publication indicates that high purity ^{123}I can be produced on a biomedical cyclotron using an enriched xenon target (171). This lack of availability has prevented the extensive study of neuroreceptors such as the muscarinic receptor with IQNB. It appears that a breakthrough in the three following technologies must be made before single-photon emitting radiotracers can be used routinely in research technology:

1) Biomedical cyclotrons can be used to prepare pure (^{124}I free) ^{123}I either alone or with a mass purification system.

2) ^{99m}Tc labeling technology is developed that allows the preparation of high receptor affinity analogs.

3) Instrumentation, especially a ring-type machine, is available to make maximum use of the emitted radioactivity.

There have been encouraging preliminary reports in all three of these areas especially in instrumentation, but to date the positron-emitting radiotracers still have an advantage. However, it is safe to say that many of the advances in receptor-binding radiotracers for external imaging have come from the pioneering work with ^{131}I and ^{123}I.

With further advances in cyclotrons, ^{99m}Tc labeling technology, and the further proliferation of the new ring instruments, single photon-emitting,

receptor-binding radiotracers will become a routine research tool if not a valuable diagnostic test.

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6
Positron Emitting Tracers in Studies of Neurotransmission and Receptor Binding

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I. INTRODUCTION

A. The Receptor and the Agonist - The Lock and the Key

Almost any biological activity within the organism is mediated after triggering of specific receptors on the cell surface or within the cell by highly specific receptor ligands i.e. transmitter molecules, hormones or drugs. The receptor-ligand complex is basically as old as the key and the lock. Other scientists beside pharmacologists, have also used this analogy. Immunologists

have used the concept to describe the interactions of antibody and antigen, and biochemists have used it for the description of the enzyme - substrate system. Vital transport functions such as the delivery of oxygen to hemoglobin from the lung to distant tissue or the movement of a metal as complex with endogenous proteins may also be looked upon as the lock and the key.

In pharmacology the analogy of the receptor and the agonist to that of the lock and the key became evident early. These receptors are usually fixed and may be up- or down-regulated as a function of the mobile agonist. Thus, the lock and the key may give a variety of effects. The receptor ligand interaction is usually reversible but may also become irreversible and ligands may act competetively or non-competetively. The same ligand may have both agonist and antagonist effects. The lock and the key concept as such is simple but the whole picture can be very complex. Thus in order to fully understand the nature of receptor interactions, much more knowledge has to be generated and new methods for such studies are highly welcome.

B. Receptors and Receptor Binding

The concept of the receptor was discussed by Ehrlich at the beginning of the century (1). Somewhat later, the concepts of neurotransmission and the neuroreceptor were formulated. In the last decades the understanding of receptor physiology has improved significantly. The number of known endogenous compounds acting in the neurons as stimulants is greater than 50 (2).

C. The Synaptic Transmission

The basic features of the synaptic transmission have been studied in some detail. A general scheme of various processes within a neuron is exemplified by the dopaminergic neuron in Figure 1.

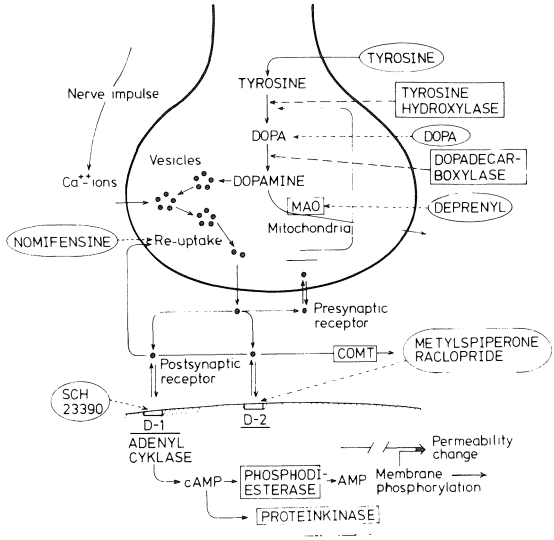

Figure 1. Schematic presentation of a dopaminergic neuron including amino acid transport, neurotransmitter synthesis, neurotransmitter metabolism and the post- and presynaptic receptors.

Since most of the neurotransmitters are unable to penetrate the blood brain barrier (BBB), they are synthesized within the brain. Peptide hormones are synthesized within the cell soma and transported as propeptides to the nerve terminals. Catecholamines, acetylcholine and the amino acid transmitters are synthesized within the nerve terminal and stored in the vesicles. On electric stimulation the synaptic vesicles release their contents into the synaptic cleft causing calcium ions to enter the terminal subsequently depolarizing the nerve terminal (Figure 2). The released neurotransmitter diffuses across the synaptic cleft, and binds to the postsynaptic membrane and/or to the postsynaptic receptor, triggering a series of events. The action of the endogenous neurotransmitters can be imitated by drugs of the agonist type. An antagonist will decrease the function of a receptor. The neurotransmitter or the neuromodulator may also interact with presynaptic receptors (3,4) on its own or with other presynaptic terminals.

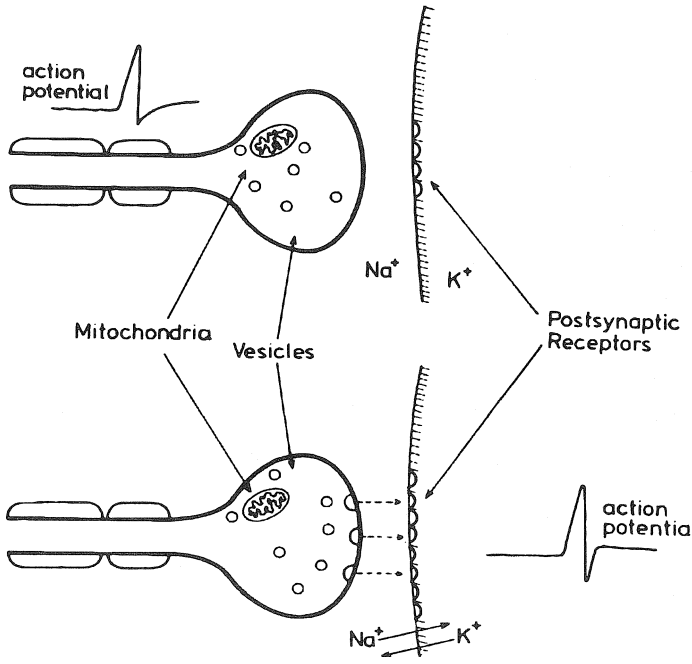

Figure 2. Scheme showing a model for synaptic transmission. The action potential at the presynaptic nerve-ending releases the transmitters in the synaptic cleft and produces the action potential at the postsynaptic terminal.

The neurotransmitter may, after release from the receptor, be deactivated by enzymes to produce metabolites in the cytoplasm or in the membranes. Alternatively it can be recycled by a re-uptake process and stored once again in the vesicles.

Many of the neurotransmitters and the neuroreceptors identified in mamalian brain, for example by autoradiography or biochemical methods, are located in the gray matter, where nerve terminals and cell bodies are concentrated. In white matter receptors are difficult to find since they are mostly located in the neuronal membrane and the cytoplasm. Receptors of the opioid, or the cholinergic muscarinic type have however been observed

intracellularly and intraaxonally (5,6). The neuronal membrane of the soma, dendrites or the nerve terminals are however the main location for most receptors.

A more detailed model of the structure of the receptor is that it is usually composed of a recognition site (the binding site) and regulatory proteins and/or enzymes e.g. adenylate cyclase. The binding of the neurotransmitter to the recognition site creates conformational changes resulting in an opening of ion channels and / or synthesis of second messengers such as cyclic 3-,5-adenosine monophosphate or inositol phosphate (7,8). The binding of a transmitter as an agonist to the receptor structure causes transmission, starting a series of one or several events to the second messenger systems within the cell. The neuroreceptor can be said to work like a transducer between the transmitter and the intracellular system. The effect of antagonist binding is to inhibit the effect of the receptor but cause no significant secondary processes. Within the nerve cell the so called second messengers are compounds which are synthesized as a result of the triggering of ligand - receptor interactions and subsequently influence the cytoplasmic and membrane enzymatic activity. Many details of these processes are still unknown.

D. Quantification of Receptor Binding

A great impact on our knowledge of receptor binding has been made by the use of substances that selectively interact with certain limited structure elements on the receptor macromolecules. However, our knowledge regarding these macromolecules constituting the receptor molecule is still limited. Such knowledge of the neuroreceptors is mainly based on various types of *in vivo* and *in vitro* binding experiments using various labelled drugs of the antagonist or the agonist type. The factors that determine whether or not a given molecule acts as an agonist or an antagonist is basically unknown. Obvious factors like stereochemistry and chemical constitution of the molecules are of great

importance. With more information of the receptor structure available it might be possible to improve our capability of preparing even more selective receptor ligands than those available today.

The concentration of most known neuroreceptors in the brain is of the order of pmole/g tissue. The specific binding of a given molecule to a protein like a receptor or an enzyme is expressed as the maximum binding B_{max} and by the equilibrum dissociation constant K_d. This implies that B_{max} relates to the receptor concentration and that K_d reflects the affinity of the ligand for the receptor (Figure 3).

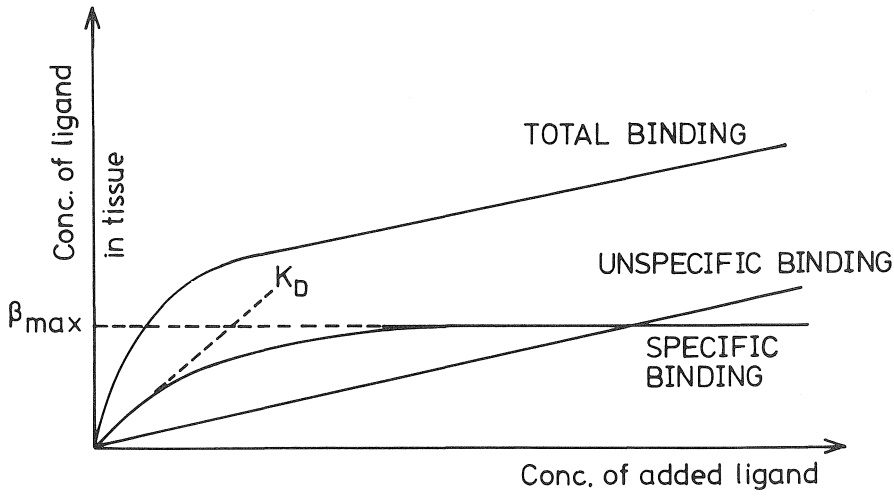

Figure 3. The plot of the ligand concentration measured as radioactivity in a specified tissue region as a function of time. In the figure the B_{max} and K_d values are measured.

E. Positron Emission Tomography

The impact of positron emission tomography, PET, as a new important imaging technique capable of studying biochemical and physiological

processes *in vivo* (9,10,11) is very much related to the development of modern detector systems and to the availibilty of a large number of tracer molecules of endogenous or exogenous origin.

Careful considerations in the design of relevant tracer molecules are opening up new and powerful multi-dimensional methods for *in vivo* studies on the kinetics of physiological and biochemical processes. The study of neurotransmission is one field which has received particular interest within this area. The characterisation of receptor binding has become a very important part in such studies. Further important contributions are expected within this field, since the method of utilising short-lived positron emitters such as ^{11}C, ^{13}N and ^{18}F gives excellent opportunities of working with compounds of high specific radioactivity. This will thus allow studies using a very low amount of mass with minor and/or neglible physiological effects or problems caused by saturation of the biological process of interest.

The main approaches in visualizing neurotransmission *in vivo* especially regarding receptor binding and neural function are by using the following types of labelled tracers:

1. Endogenous transmitters.
2. Endogenous substrates for neurotransmitters
3. Selective receptor binding ligands
4. Selective molecules visualising other processes of the neuron such as the uptake system, reuptake system, and enzymatic metabolic systems either for neurotransmitter synthesis or for the degradation of the neurotransmitters.

The development of a number of labelled tracer molecules has made possible the visualization of selective processes in complex biological systems like the brain. This can be performed using various molecular tools, as exemplified by the compounds in oval frames in Figure 1. This involves the use of various endogenous or exogenous substrates for enzymes and/or

receptor ligands to study the processes of neurotransmisson *in vivo*. The combination of such labelled compounds with improved statistical methods such as multivariate analysis (12) might in the future give a rational description of the function or malfunction of complex biological systems.

Factors such as the distribution and selectivity as well as the choice of the appropriate radionuclide, the position of labelling, the stereochemistry of the molecule and the mode of administration are important in studies using radiolabelled tracers. Blood brain barrier permeability, disposition of the tracer in the body and type of binding in the tissue are other important considerations to be made before choosing radiolabelled tracer-molecules for studies using PET.

In this chapter some of the main features in investigations utilizing PET in studies on neurotransmission and receptor binding will be discussed. So far the main part of the investigations have been focused to the brain, and the examples are chosen from studies on this organ. The approaches used for the receptor studies can, however, be generally applied to other biological systems.

F. Positron Emission Tomography in Studies of Neurotransmission

The main approaches regarding neurotransmitter studies are indicated in Figure 1 exemplified by the dopaminergic neuron. For the dopaminergic neuron, precursors for neurotransmitter synthesis such as L-DOPA (13) or precursor analogues such as 6-Fluoro-DOPA (14,15,16) labelled with the appropriate radionuclide are used. Another approach is to use selective dopamine receptor ligands with various profiles for receptor binding (17,18,19). The re-uptake system of monoaminergic transmitters as in i.e. dopamine has been visualized with PET using nomifensine (20,21). Other ways of visualizing the function of the neuron is to use selective enzyme substrates or irreversible enzyme inhibitors. (22). Studies on various synthesising as well as degrading enzymes are thereby of relevance. This

approach is discussed in another chapter (Fowler).

The utilization of the aforementioned tools gives us methods with the potential for measuring normal as well as altered neurotransmission which may have resulted in abnormalities regarding

- storage and release of the neurotransmitters
- metabolism and reuptake of the neurotransmitters
- receptor binding
- the coupling of second messengers to the neuroreceptor.

II. POTENTIAL APPROACHES TO IN VIVO VISUALIZATION OF NEUROTRANSMISSION

A. In Vivo Visualization of Neurotransmitters

In transferring knowledge from *in vitro* based information to applications *in vivo* using the PET method, several new aspects have to be kept in mind. The rapid turn-over *in vivo* of a neurotransmitter presents us with special problems. In studies using the neurotransmitters themselves, the passage through the blood brain barrier (BBB) is usually low. However an interesting approach is to use carrier mediated transport systems such as the dihydropyridine-pyridine salt as a carrier of various compounds of interest (23). Use of neurotransmitter precursors may overcome the penetration problem, but the observed process may be even more complicated due to the formation of metabolites. Before selecting an appropriate strategy in the visualization of the turn-over of the neurotransmitter the rate limiting step should be considered. The use of a short-lived tracer such as ^{11}C-L-DOPA when transport over the blood brain barrier is the rate-limiting step might not be a proper choice, if the aim of the investigation is to get information of the functions in the synaptic cleft. In this case the use of an analogue like 6-^{18}F-L-DOPA might be a better choice since the biological rate determing step is comparable to the half-life of the physical half-life of the radionuclide.

B. In Vivo Quantification of Neuroreceptor Binding

The biochemical criteria and evidence for a receptor-ligand binding *in vivo* using PET are the following:

- the binding is *saturable and selective*
- the binding is *stereoselective*
- the ligand-receptor binding has *kinetic binding constants of the same magnitude as those observed from in vitro binding studies*
- the ligand binding is described by various *competition experiments in order to identify multiple binding sites*

A variety of ligands have been used in vivo (Table 1).

Table 1. List of Some Ligands/Drugs Which Have Been Used in PET Studies.

RECEPTOR	RADIOLIGANDS	REFERENCE	COMMENT
Dopamine	^{11}C-N-methylspiperone	17	D2/5-HT2
	^{18}F-NMSP	24	D2/5-HT
	^{18}F-spiperone	24	D2/5-HT2
	^{18}F-haloperidol	25	
	^{18}F-benperidol	24	
	^{11}C-clozapine	26	D2/5-HT2
	^{11}C-raclopride	18	D2
	^{11}C-eticlopride	18	D2
	^{11}C-SCH 23390	27	D1
	^{11}C-pimozide	28	D2
	^{11}C-chlorpromazine	29	
	^{76}Br-spiperone	30	D2/5-HT2
Serotonine/5HT2	^{11}C-ketanserin	31	
	^{11}C-methylketanserin	31	
	^{11}C-methylbromo-LSD	32	
	^{11}C-zimelidine	Hartvig/ Unpublished	
Choline	^{11}C-choline	33	

Table 1 (cont'd.) RECEPTOR	RADIOLIGANDS	REFERENCE	COMMENT
Muscarinic	^{11}C-QNB	34,35	
	^{11}C-dexetimide	36	
	^{11}C-scopolamine	37	
Nicotinic	^{11}C-nicotin	38,39,40	
GABA	^{11}C-GABA	41	
	^{11}C-MK801	33	
Benzodiazepine	^{11}C-Ro-15-1788	42,43	
	^{11}C-suriclone	44	
	^{11}C-PK 11195	45	
Adrenergic	^{11}C-pindolol	46	
Opiates	^{11}C-carfentanil	47	
	^{11}C-diprenorphine	48	
	^{18}F-acetylcyclofoxy	49	
	^{11}C-pethidine	50	

C. Models for Quantification of Receptor Binding

All quantitative analysis of receptor binding *in vivo* relies on relevant tracer kinetic models. Changes in receptor binding due to alterations in receptor number (concentration) B_{max} or affinity K_D must be distinguished from other non-receptor processes such as blood-flow, blood brain barrier transport, non-specific binding and metabolism.

Two main approaches in modelling receptor binding using PET-data have been applied: the *kinetic* and the *equilibrium* models.

In the *kinetic* model equilibrum conditions are not present and the approach is dependent on the assumption that the receptor binding relates to association and dissociation of a ligand over time. This approach has been applied for example in studies utilizing a 3-compartment model, i.e. ligand in blood, ligand in non-receptor regions and ligand in the receptor compartment as shown in Figure 4. Considerable efforts have also been made in the mathematical treatment of data to fit the models which are made to give an *in*

vivo quantitative measure of the receptor characteristics (51-56).

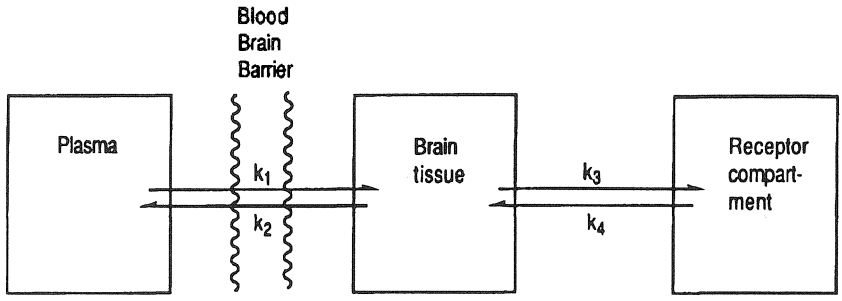

Figure 4. A schematic presentation of a 3 compartment model which was used for quantification in receptor-ligand binding in the brain.

In the *equilibrium* model, it is assumed that at a certain time after administration an effective equilibrium is reached. By performing at least two administrations at different specific radioactivities the B_{max} and K_d can be obtained by Scatchard analysis. In experiments where 5 studies were performed on each subject, B_{max} or K_d was obtained by a saturation analysis according to Figure 3. Hill plots were applied to assure that just one type of binding was present (57). These methods resemble closely the ones used for *in vitro* studies.

Both approaches require at least that the PET experiments are performed at various levels of receptor occupancy. Only the ratio of receptor concentration (i.e. receptor number) and affinity can be obtained using the kinetic model. The data in the models can be handled in various ways, for example, by curve fitting routines (54,55) or by graphical methods (58,59). All

the methods rely heavily on the accuracy of the input function, that is the time course of the concentration of the compound in arterial plasma which also must be compensated for changes in blood flow, metabolism and receptor affinity of metabolites - labelled or non-labelled reaching the target site. Various approaches have been evaluated in order to circumvent problems due to variations in the input function caused by trapping of metabolites and time dependent ligand release (54).

PET as a radiotracer technique might thus allow a detailed kinetic pharmacological interpretation or characterization of the tracer in the intact tissue section by section in addition to its utilization in the studies of the function of neurotransmission and receptor binding. Receptor binding as measured by PET may also indicate the dynamic action of a receptor ligand and allow for contributions on pharmacodynamic effects of the ligands which might be of clinical importance.

One example of this kind is the receptor binding properties of the antipsychotic drug, clozapine as evaluated with PET which made possible suggestions concluding its clinical advantages (60). It has also been possible by means of PET to draw conclusions on the mechanism of action of the neurotoxin N-methyl-phenyl-1,2,3,6-tetrahydropyridin (MPTP) (61) (Figure 5)

The short-lived positron-emitting radionuclides can also be used in applications of the ordinary tracer type, comparable to *in vitro* binding studies. Comparison of data from *in vivo* and *in vitro* autoradiography may further extend the applications of the technique (62,63,64).

D. Specific Radioactivity

The tracer methodology using positron emitting radionuclides like ^{11}C,

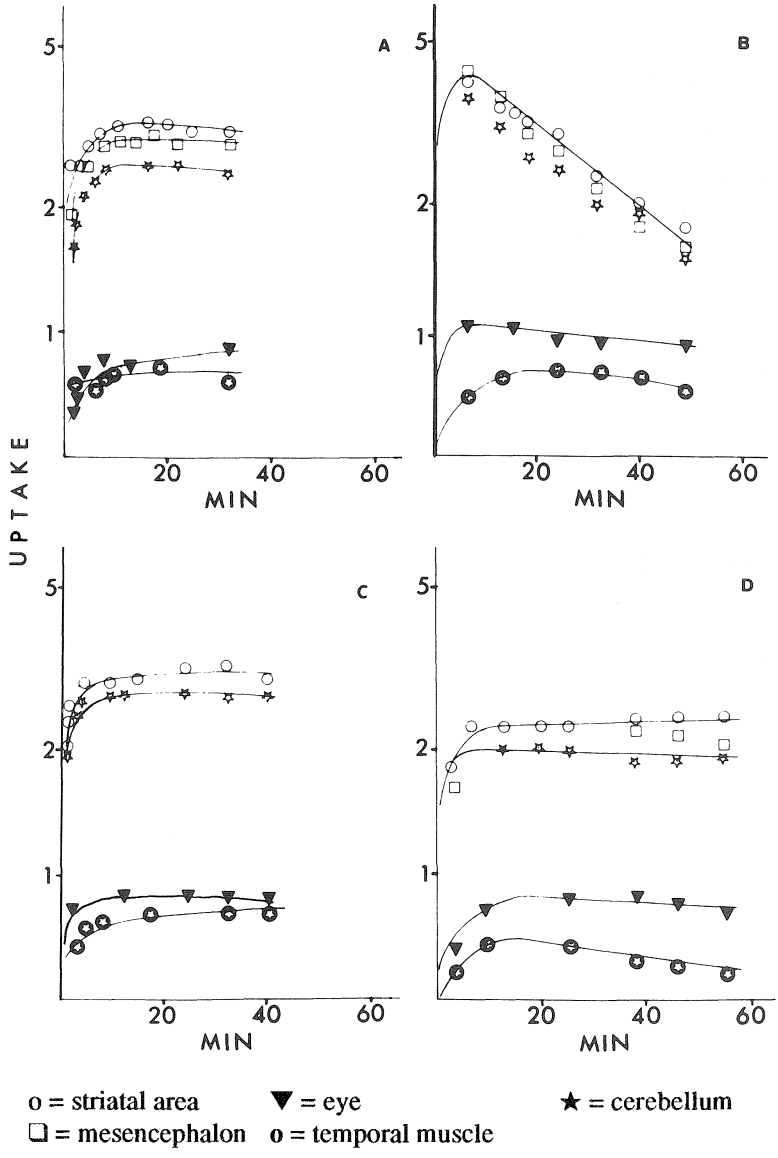

Figure 5. The uptake kinetics in monkeys of ^{11}C-MPTP after various pretreatment A) control B) pargyline C) clorgyline and D) nomifensine.

^{13}N and ^{18}F has one very important quality. The compounds can be obtained having high specific radioactivity thus making it possible to perform pure tracer studies in most known biological processes with neglible disturbances of the biological system. In studies at various concentrations i.e. at various receptor occupancies, problems might arise due to pharmacological effects when administering larger doses. Several approaches have been applied in order to circumvent this problem such as the use of haloperidol as a competitive drug for the dopamine receptor with studies using ^{11}C-N-methylspiperone (NMSP) (52), and the use of compartment analysis to describe the blood-brain barrier transport and receptor binding using combinations of reference organs as internal standards (54).

E. Studies Using Labelled Analogues

The use of homologues as ligands in order to observe a given process by means of PET can be valuable. The use of spiperone analogues is one example (24). The receptor affinity of spiperone is higher than that of NMSP, but the penetration of the blood brain barrier is apparently a rate-limiting step. Since NMSP is more lipophilic it penetrates the BBB more efficiently and is therefore a more interesting ligand as a tool for in vivo characterisation of receptor binding in the brain. The need to optimise such *in vivo* properties has created a search for other alkyl substitued spiperone ligands. Another perspective in this aspect is the search for ^{18}F-alkyl substituted spiperone derivatives since this might offer compounds having higher specific radioactivity. In such studies the utilization of the longer half-life of ^{18}F enables us to get better data on the ratio of the receptor binding to the nonspecific binding.

F. Animal Models

The use of animal models for the evaluation of potential tracers is one

important strategy. This could of course imply the use of various pathological animal models such as induced tumours, induced neurological disorders or ischemia. Careful interpretation of the data is necessary, particularly in the direct extrapolation of results to humans due to species differences. It has been possible with PET to study the metabolism of the neurotoxin MPTP in the Rhesus monkey brain and its mechanism of toxicity has been enlightened (61) (Figure 5). Using MPTP as a neurotoxin, a unilateral lesion in Rhesus monkey brain was achieved and used as an animal model for Parkinson's disease. PET-studies were performed using a multi-tracer approach; ^{11}C-raclopride, ^{11}C-deprenyl, ^{11}C-nomifensine to evaluate the neurotoxic effect on different functions of the dopaminergic neurone (Figure 6; see color plate). It was further shown that there was no obvious change in MAO activity before and after the lesion (65).

G. In Vitro Applications

The short-lived positron emitting radionuclides are not only of interest in in vivo studies utilizing the annihilation photons as in PET. These radionuclides also have other properties which make them of interest for *in vitro* studies. Using the β-particle ordinary autoradiography has been performed using ^{11}C-compounds, also in relation to PET-studies (62,63,64) with a resolution comparable to the more frequently used ^{3}H and ^{14}C radionuclides.

III. STRATEGIES IN SELECTING RADIOLABELLED TRACERS

A. In Vivo Kinetic Considerations

Due to a very rapid *in vivo* metabolism in the blood and slow penetration through the BBB, it is not possible to perform PET-studies using endogenous compounds as the neurotransmitter by direct intravenous administration. This is also obvious for neuropeptides like metenkephalin and substance P labelled

with ^{11}C (66). In these studies the radioactivity in the brain emanated mainly from labelled metabolites formed in the blood. More stable analogues of the enkephalin peptides apparantly penetrated the BBB without being metabolised. In this case PET might be an excellent screening method for studies on the distribution of intact peptides to the effected compartment.

Studies of slow transport processes *in vivo* with PET may be difficult with the short-lived radionuclides like ^{15}O, ^{13}N and ^{11}C. In such a case ^{18}F is an interesting alternative. The reason for that is that there must be a correlation between the biological half-life of the tracer and that of the physical half-life of the radionuclide.

Extensive metabolism of a radiotracer molecule during the course of the PET-study may cause severe problems in the interpretation of data. This is for example the case in studies of neurotransmitter turn-over from various labelled precursors. Complementary techniques may be used in order to analyse the composition of metabolites in the blood by means of for example TLC or LC. Thus studies on the endogenous neurotransmitter turn over in the brain have so far been restricted to gross effects with little possibility of discriminating processes such as formation or degradation of the tracer molecule. A promising way to overcome these difficulties is to label the tracer molecule in an alternative position in order to observe the differences between the two processes. An example of this is the use of 1-^{11}C- and 3-^{11}C-labelled L-DOPA in order to investigate the dopamine synthesis within the brain (67,68,69). Using 1-^{11}C-DOPA the PET-images will contain information on labelled DOPA and carbon dioxide, however in the case of 3-^{11}C-DOPA the same images will now contain information on labelled DOPA and dopamine (and its metabolites) (Figure 7; see color plate).

Studies with radio-labelled receptor ligands, including exogenous ones used as drugs, are less hampered by the problem of extensive metabolism during PET-investigations. Most of the compounds used in PET-studies of the

brain are lipophilic allowing rapid BBB-transport. The fraction of labelled metabolites is usually small and in many cases they are more polar thus causing less problems with regard to their brain-uptake. However this problem has to be investigated for each tracer, one example being a study using ^{11}C and ^{18}F-NMSP (70) .

B. Properties of the Tracer Molecules

1. CHOICE OF RADIONUCLIDE

It is very important if for example a hydrogen atom in L-DOPA is substituted with fluorine, that the new molecule exhibits similar biological properties. Differences in processes like peripheral decarboxylation and amino acid transport as well as parameters like volume of distribution must always be considered very carefully.

2. STEREOCHEMISTRY

The stereochemical purity of the preparation is another factor which has to be determined. It is of particular interest if two enantiomers can be used, one having low selectivity and the other a much higher selectivity for the process of interest. An example is shown in Figure 8 (see color plate), where the two enantiomers of ^{11}C-nicotine have been used. The brain uptake of the active enantiomer increased dramatically after the blockade, whereas the uptake of the inactive form remained at the same level (38). The enantiomers were used in order to avoid differences caused by changes in cerebral blood flow or pH, since the lipophilicity and the pK for the enantiomers are assumed identical.

One has always to be careful in applying this approach, however, since the enantiomers may have different selectivity or metabolism. Nicotine has a pK_a matching the physiological pK_a and is thus also a potential marker for physiological pH.

3. POSITION OF LABELLING

In order to control problems arising due to metabolism, the possibilty of labelling a ligand in various positions or with different radionuclides might give relevant information on problems due to labelled metabolites. Using the same ligand but with the label in various positions is one way of obtaining information with PET about the contribution of metabolites to the obtained data. Compairing labelled NMSP with either ^{11}C in the N-methyl group or with ^{18}F in the phenyl ring in the butyrophenone structure clearly indicated that metabolites do not contribute to the specific receptor binding (70).

In PET-studies using dimethylphenethylamine the label has been put in two different positions in order to distinguish between binding and metabolism. Putting the label in the N-methyl group indicated that a fast metabolism occurred yielding a polar metabolite which was trapped within the brain. Quite the opposite happened when the label was placed in the phenethyl group. In this case a more lipophilic metabolite was obtained which easily was eliminated from the brain. This matter is discussed in more detail in the chapter by J. Fowler.

4. MULTIPLE ISOTOPIC LABELLING

In some situations it might be of interest to produce compounds which are labelled with more than one isotope. For example, when deprenyl is labelled with ^{11}C in the N-methyl group and the protons in the methylene group have been replaced by deuterium atoms a significant change in brain uptake is observed. This change is most likely an isotopic effect caused by the rate determing enzymatic step (71). It is also possible to perform syntheses using double isotopic labelling such as for deuterated ^{11}C-alkyl iodides.

IV. SYNTHESIS OF SHORT-LIVED LABELLED TRACERS APPLIED IN NEURORECEPTOR AND NEUROTRANSMISSION STUDIES.

Most of the ligands so far applied in receptor binding studies with PET have been synthezised by alkylation reactions of the appropriate dealkylated precursor. The majority of these compounds have been prepared by using ^{11}C-methyl iodide and the corresponding desmethyl compound and labelling in polar solvents such as dimethylformamide, dimethylsulfoxide or acetone (72). In a few cases ^{11}C-formaldehyde has been used in a reductive type of alkylation. This reaction is of special interest since it can be performed in an aqueous medium.

More complex chemistry that involves multiple steps including organic and enzymatic synthesis has been used in order to prepare other compounds such as the substrates for the neurotransmitters DOPA and 5-hydroxytryptophan. This is also true when discussing ^{18}F-labelling where several approaches have been applied. In the case of the synthesis of ^{18}F-alkylspiperones one strategy has involved the preparation of the appropriate alkyl fluoride followed by an N-alkylation reaction.

V. APPLICATIONS OF PET IN NEUROTRANSMISSION AND RECEPTOR BINDING STUDIES

A. Dopamine and the Dopaminergic Receptors

The catecholamine dopamine is specifically located in hypothalamic and brain stem nuclei which have projections into the caudate nucleus, putamen, nucleus accumbens, prefrontal cortex and the spinal cord. In pathological states, dopamine deficiency has been observed *i.e.* in Parkinson's disease (73). Alterations in the dopamine neurotransmission has also been implied in schizophrenia due to the observed improvements of symptoms which have been observed after treatment with various dopamine receptor antagonists (57,74,75). Dopamine receptors might also play a role in a number of other

neurological and psychiatric conditions like Huntington's chorea (76,77) Tourette's syndrome, dystonia and tardive dyskinesia (74,78,79).

B. Neurotransmitter Precursors

In the dopaminergic neurons L-DOPA is produced from tyrosine via a hydroxylase catalysed reaction (Figure 1). An enzymatic decarboxylation leads to the formation of dopamine from L-DOPA, which is subsequently stored in presynaptic vesicles.

In PET-studies investigating the synthesis of dopamine, the precursor has to be labelled in such a way that the label will remain on the transmitter even after its conversion to dopamine. On the other hand if it is of interest to observe the production of the neurotransmitter itself it would be necessary to place the label in such a way that the metabolite would be labelled. A variety of neurotransmitters are now being investigated using PET (Table 2).

Table 2. List of Substrates for Neurotransmitter Synthesis Being Applied in PET Studies.

RECEPTOR	SUBSTRATE	REFERENCE
Dopamine	^{18}F-6-fluorodopa	14
	^{11}C-Dopa	67,68,69
	^{11}C-tyrosine	80
Serotonine	^{11}C-5-0H-tryptophan	81
	^{11}C-tryptophan	81
Choline	^{11}C-choline	33
Opioids	Enkephalines	66
	Substance P	82

[β-^{11}C]-L-DOPA is thus a good choice for the dopamine synthesis and preliminary studies using this tracer are now in progress performed by means of PET. Using β-^{11}C-L-DOPA (67,68,69) in PET-studies on monkeys and humans the brain radioactivity in the striatal area increased after pretreatment

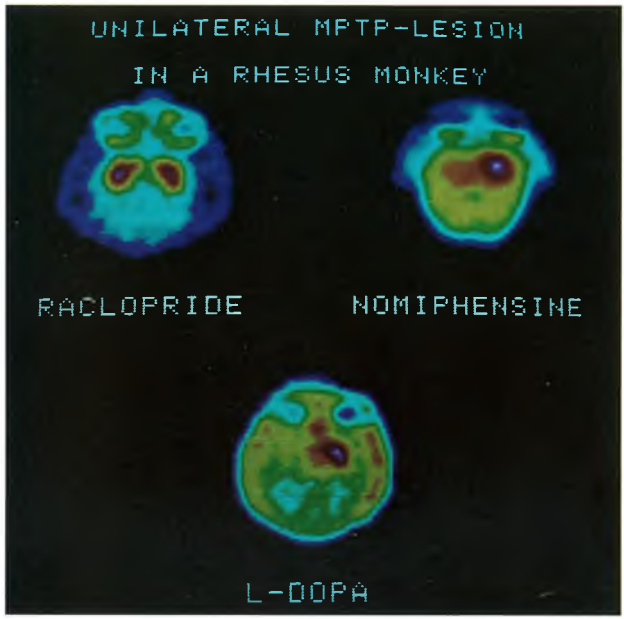

Figure 6.6. Multitracer PET studies to elucidate the neurotoxic effect of MPTP after a unilateral lesion—^{11}C-nomifensine for the re-uptake dopaminergic site, ^{11}C-raclopride for the D_2-receptor binding, and ^{11}C-L-DOPA for neurotransmitter synthesis. (See page 237.)

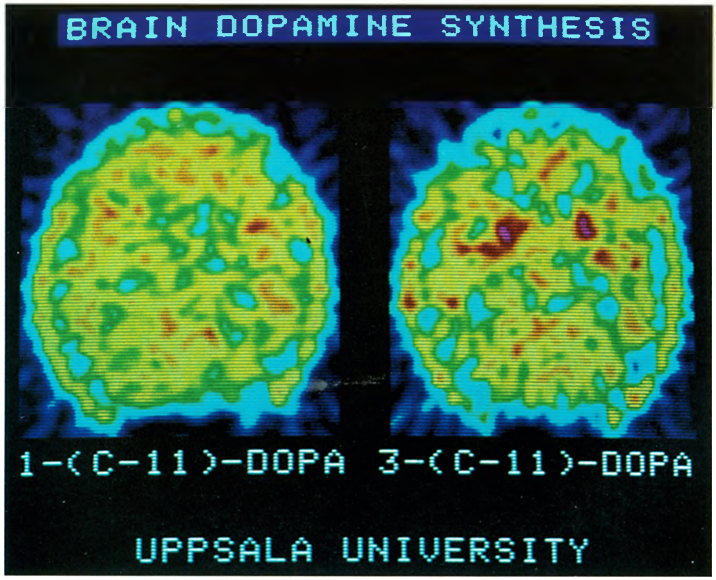

Figure 6.7. PET studies using carboxylic and β-^{11}C-labeled L-DOPA, an example of the importance of molecular design in labelling appropriate tracer molecules. (See page 238.)

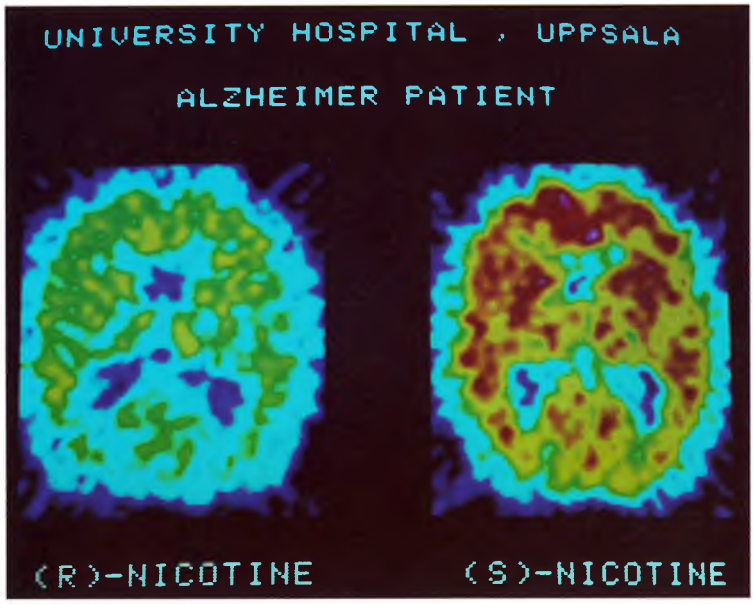

Figure 6.8. PET images of a patient with Alzheimer's disease using both enantiomers of ^{11}C-nicotine. (See page 239.)

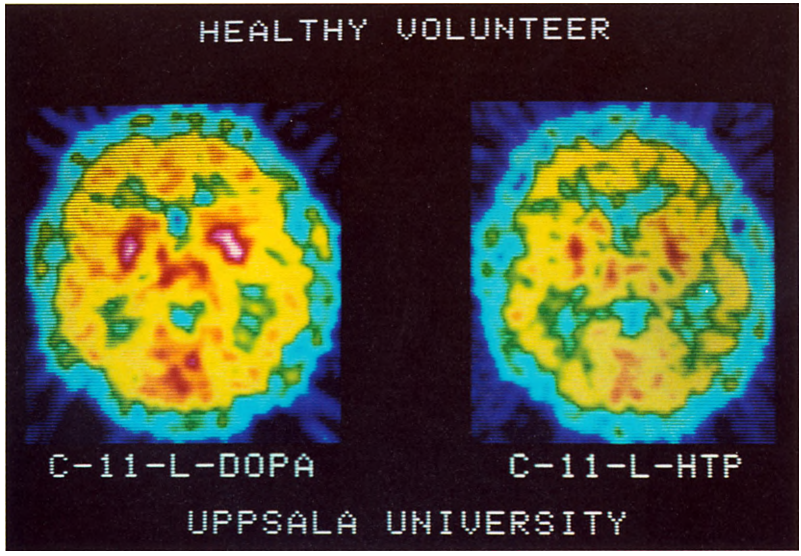

Figure 6.9. A comparison between the distribution of two neurotransmitter substrates, 5-hydroxytryptophane and L-DOPA, labelled with ^{11}C in the 3 position, indicating the synthesis of the corresponding neurotransmitter. (See page 243.)

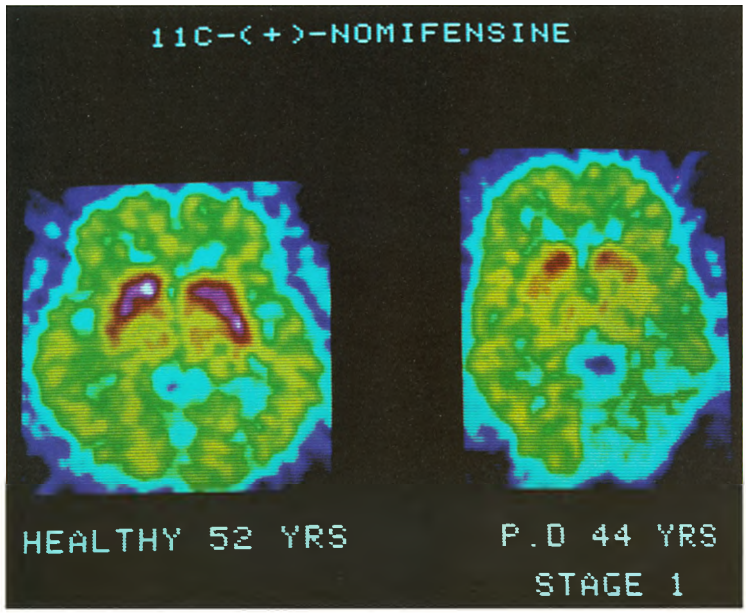

Figure 6.10. A re-uptake ligand, ^{11}C-nomifensine, used in PET studies of an age-matched Parkinsonian patient and a healthy volunteer. (See page 245.)

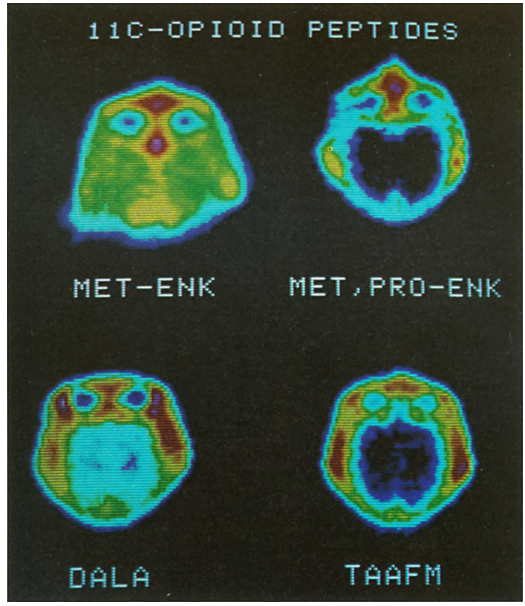

Figure 6.13. PET images of ^{11}C-metenkephaline and three enkephaline analogues in the rhesus monkey: an example of the complicated situation when studies include uptake of metabolites. (See page 252.)

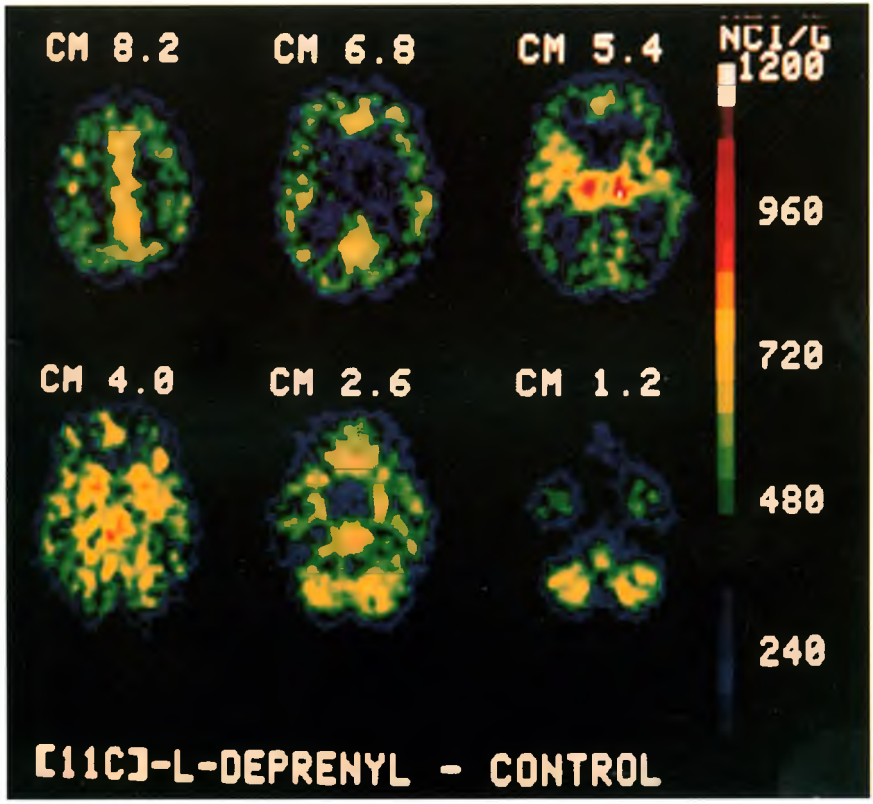

Figure 7.10. PET images taken after injection of [^{11}C]-L-deprenyl at different levels above the canthomeatal (CM) line showing high radioactivity accumulation in the cingulate gyrus, basal ganglia, thalamus, and brain stem. (See page 286.)

with a peripheral decarboxylase inhibitor such as benserazide or after a single large dose of unlabelled L-DOPA and 5-HTP supporting the view that a selective process was observed (Figure 9; see color plate).

The use of the analogue 6-^{18}F-L-DOPA has been shown to be a promising substitute for DOPA. The use of ^{18}F might in this case be appropriate since the rate determining decarboxylation step doesn't seem to be significantly affected by the ^{18}F-substitution. Using this tracer in studies of normal and abnormal neurotransmission, i.e. Parkinson's disease (14,15) a significant decrease in radioactive uptake has been observed indicating a lower synthesis rate for dopamine from the precursor. However, these studies illustrate the difficulties in the interpretation of complicated PET-data. Is the decreased uptake due to a change in amino acid transport or does it reflect decreased dopamine synthesis, or is it a combination of both effects? Wurtman et al (83) addressed these problems.

C. Receptor Ligands

In the pioneering work of visualizing dopamine receptors in humans (17), ^{11}C-NMSP was used. The ligand has also some affinity for the serotonin receptor of the HT2-type. Later ^{11}C-labelled substituted benzamides of various types such as raclopride have proven to be very selective for the D2-receptor (84). These and a number of other very selective dopamine ligands have then been used to address different problems within the biomedical field using PET. Applications of labelling receptor ligands with gamma emitting radionuclides to be used in single photon spectroscopy (SPECT) have also been performed. This is dealt with in another chapter (Kung).

1. SUB-CLASSES OF THE DOPAMINE RECEPTOR

In vitro characterisation of the dopamine receptors has revealed at least

two subtypes of receptors i.e. the D1 and the D2 receptors. The D1 receptor seems to be associated with adenylate cyclase, however the function of the cyclase and its role in the formation of cyclic AMP is at present not known. The D2 receptor is not linked to adenylate cyclase. There is also a presynaptic dopamine receptor the autoreceptor, which is involved in the control of dopamine release from the vesicles.

A large number of receptor ligands particularly for the D2-dopamine receptor have been applied in PET-studies (cf. Table 1). However the large majority of studies have been performed using NMSP. The ligands have been shown to fulfill the selective receptor-binding criteria, although some of them also have affinities for other receptor types. Several of the ligands have also been applied in clinical studies. In short, studies using NMSP show a substantial decrease in the dopamine and serotonin receptor number with increasing age, and was most pronounced in men (85). Using the same ligand an up-regulation of dopamine receptors was observed in the striatum in early cases of Parkinson's disease (86,87). On the other hand dopamine receptors were almost absent in patients with Huntington's chorea (86).

A great controversy surrounds the question if there is an alteration in dopamine receptor binding in patients with schizophrenia. Wong et al (75) showed an up-regulation in receptor binding using NMSP whereas Farde et al (57) using raclopride and Andersson et al (79) using NMSP were not able to show any difference. Inclusion criteria for the patients and different calculation methods may be responsible for the different results. On the other hand no up regulation of dopamine binding was observed in other studies on patients with tardive dyskinesia (79).

A method used on a clinical basis utilizes either raclopride or NMSP for the assessment of the dopamine receptor number in pituitary tumours (88). Increased dopamine D2 receptor binding evaluated with PET gives relevant indications for the treatment of the tumour and the effect of treatment can also

be evaluated with PET (89,90).

Receptor binding characteristics have been estimated for different dopamine receptor antagonists (19,65). Different binding rate constants have been found for different antipsychotic drugs which may be of consequence for their clinical effects (19). It has also been shown that antipsychotic drugs in therapeutic doses occupy up to 90% of the D2 receptors (91)

The possibility of performing studies using sub-selective receptor ligands such as D1 and D2 receptors is another interesting approach. Ligands for studies of D1-dopamine receptor binding (27) are also now available. Studies have shown that there is a difference in the localisation of the D1 and D2 receptor binding in the brain as measured with raclopride (57,91).

The use of other selective ligands with profiles for the presynaptic dopaminergic receptor has given us the potential for multivariate strategies by the use of various selective receptor ligands in order to enlighten our understanding of the functions of the neurons in the living system. 2-aminotetralines, selective for the presynaptic receptors (92) have in preliminary PET-studies shown to be selective for the dopamine D2 receptor.

D. Reuptake Ligands

Nomifensine labelled with ^{11}C has been used in PET-studies to visualize the dopamine reuptake system (20) The ligand showed high affinity and selectivity for the monoaminergic reuptake sites in the brain (Figure 10; see color plate). The same studies showed that there was no interaction with the imipramine and spiperone binding sites. In humans there was also an age dependant decrease in nomifensine binding in the striatum as is also the case with age-matched patients with Parkinson's disease (21). A good correlation has also been shown between ^{18}F-L-DOPA and nomifensine uptake in patients with Parkinson's disease (21).

E. Serotonin and Serotonergic Receptors

Serotonin receptors are found in high concentrations in the brain stem, raphe nuclei and the neurons projecting to cerebral cortex, striatum, hippocampus, hypothalamus cerebellum and the spinal cord. The serotonin-receptor interaction is hetereogenous since serotonin interacts with multiple binding sites (93,94,95). The 5-HT_1 receptor is coupled to adenylate cyclase and has a high affinity for serotonin agonists and ergot drugs causing changes in the motor functions, mood and sleep pattern. A second type of serotonin receptor is the 5-HT_2 type which has affinity for butyrophenones such as haloperidol and spiperone. Development of several ligands for studies of serotonin receptor binding in PET-studies, as listed in Table 1, are now in progress.

Serotonin turnover has been implied to be an important factor in several psychiatric disorders such as affective disease and insomia. The possibility to study the turnover of the neurotransmitter after administration of a suitable labelled precursor is therefore one important approach to understanding the disease. Promising results to evaluate a selective process both in monkey and humans has been shown using β-^{11}C-5-hydroxytryptophan (81,96, Langstrom work in progress) (Figure 9). A selective accumulation of radioactivity was observed in the frontal cortex and the striatal area as well as other regions known for high receptor densities. Ligands to visualize a selective serotonin re-uptake such as cyanoimipramine or zimelidine have also been used in PET-studies (unpublished results).

Many drugs used in the clinic have affinity for several receptors. Some of these are probably effective therapeutically due to their combined effect on several receptor systems. PET can advantageously be applied in the evaluation of such drugs. Using clozapine labelled with ^{11}C (26) in studies combined with pharmacological doses of receptor selective drugs it was possible to obtain relevant information about its *in vivo* pharmacological profile with

respect to both serotonin and dopamine receptor binding (19,26).

F. Benzodiazepine Receptors

The benzodiazepines are a class of compounds which have a multitude of effects and are typically used for their anxiolytic and anticonvulsive mode of action (97). They affect the function of the chloride ion channels in the GABA receptor complex in such way that the chloride conductance and hence the sensitivity of the GABA-receptor is altered (Figure 11).

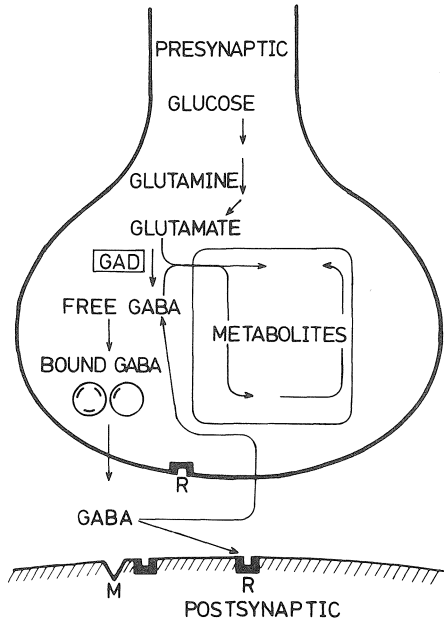

Figure 11. A schematic model of the GABA neuron.

Different benzodiazepine ligands such as Ro-15-1788, flumazenil, and suriclone labelled with ^{11}C (42,43,44) have been shown to have a similar distribution in the brain. The binding is not greatly affected by GABA or by chloride anions. This might indicate that changes in binding are caused by

intrinsic changes *in vivo* such as affinity or receptor number, or by changes due to competition with an endogenous neurotransmitter, but not directly by changes in binding itself.

Early studies using ^{11}C-labelled flumazenil, showed a high fraction of selectively bound tracer in the brain and that the radioactivity was easily displaced (42). Competetive binding experiments in man showed binding to the same type of receptor as diazepam (43).

Other interesting clinical applications for the benzodiazepine receptor ligands are the use of these ligands for the delineation of epileptic foci and for the characterisation of tumours (97). Diphenylhydantoin labelled with ^{11}C has also been tried in the diagnosis of epilepsy (98).

G. Cholinergic Receptors - Muscarinic and Nicotinic

Acetylcholine is a neurotransmitter which when released effects a multiplicity of receptors of both the excitatory and the inhibitory type. It is postulated that the fast excitatory receptor is the nicotinic receptor and that the slow excitatory receptor is the muscarinic receptor. (Figure 12)

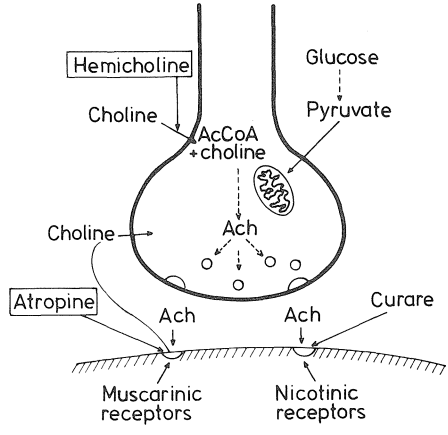

Figure 12. A schematic model of the cholinergic neuron.

Efforts have been made to estimate the turn-over of acetylcholine using PET after the administration of ^{11}C-choline and choline analogues (33). The observed uptake of radioactivity into the brain was small, however by using data from analogues it was possible to estimate the different steps involved in choline metabolism.

1. NICOTINIC RECEPTORS

One of the criteria for receptor binding is stereoselectivity. The two enantiomers of ^{11}C-nicotine were used as ligands for studies of the receptor binding of nicotine by means of PET. The brain kinetics of the radioactive uptake in four studies are summarised:

a) with the inactive (low affinity) (+)-nicotine,

b) with the active (high affinity) (-)-nicotine,

both performed with and without a peripheral nicotine receptor blocking agent infused throughout the PET-study.

The interpretation of the data is that in the control experiment the peripheral nicotine receptors trap the (-)nicotine. It was concluded that specific binding of (-)nicotine was observed. In this case the use of both enantiomers was essential, since the (+)- enantiomer was used as control in order to quantify processes such as blood flow and cerebral pH.

After a bolus injection the labelled tracer is slowly released from the peripheral binding-sites and taken up into the brain (38) (Figure 8). In preliminary studies using the ^{11}C-labelled enantiomers of nicotine a pronounced upregulation of cerebral nicotine receptors was visualized when comparing a non-smoker with a smoker (39,40).

2. MUSCARINIC-CHOLINERGIC RECEPTORS

Early in the development of PET, elegant studies were performed to

visualize muscarinic-cholinergic receptor binding in the baboon heart (34,35). The selectivity of the binding was proved by a selective dose-dependent displacement of radioactivity of the labelled tracer using atropine. However in these studies the quaternary ammonium salt methyl-QNB was used which passes poorly through the BBB. Attempts to use other selective ligands such as dexetimide labelled with ^{11}C are in progress (36).

H. Opioid Receptors

Opioids are known to produce a range of pharmacolgical effects such as analgesia and respiratory depression. Autoradiographic studies as well as PET-investigations have revealed that high densities of the brain opioid receptors are localized in the amygdala, thalamus, and caudate nucleus. Lower densities have been observed in the frontal and parietal cerebral cortex, cerebellar cortex, cingulate cortex and hippocampus. This regional distribution corresponds to *in vitro* data (47). Other PET studies using opioid ligands are listed in Table 1.

A rapid metabolism to hydrophilic conjugates together with a rate limiting BBB-passage has hampered PET-studies of opioid receptors using labelled receptor agonists like morphine and heroin (99,100). Efforts to use more lipophlic receptor agonists such as pethidine have not been successful due to rapid dissociation from the receptor (50).

^{11}C-carfentanil with a much higher binding affinity has shown uptake in the brain corresponding to the opioid receptor distribution determined for example by autoradiographic techniques (47).

In the case of PET-studies using opioid agonists such as carfentanil it is important that compounds with high specific radioactivity are used due to the pharmacological effects of even submicrogram doses of the drug. In such cases the practical limitations have to be considered and the use of ^{11}C in this case might be a second choice to ^{18}F with respect to higher specific radioactivity.

A partial opioid receptor agonist buprenorphine (48), and antagonists like acetylcyclofoxy (49) circumvent the problems of pharmacological effects given even by tracer doses of the ligands i.e submicrogram level.

Enkephalin analogues have, as is discussed in the section on neuroactive peptides, only been studied to a small extent with PET.

I. Amino Acid Transmitters

Two amino acids GABA and glycine have been shown to exert inhibitory actions in brain tissue but also participate in general reactions of intermediary metabolism in the brain (41). Even if these amino acids can be regarded as putative neurotransmitters, their widespread occurrence makes it difficult to assign them as truly neurotransmitters, but it is known that they exhibit some modulatory functions such as excitability. No PET-studies using the amino acids themselves have so far been reported.

J. Neuroactive Peptides

The list of centrally active neuropeptides is steadily increasing and a lot of knowledge has been gained in recent years. As for the amino acid neurotransmitters it is not clear whether the amino acids act as modulators or as transmitters. In either case they represent a fascinating group of centrally active compounds with many discoveries to be anticipated.

Positron emitting labelled neuroactive peptides are now available, however, a major draw-back is that the endogenous peptides such as enkephalines and substance P are very rapidly metabolized *in vivo*. The use of analogues, which are more stable to degrading enzymes and metabolised more slowly, is more satisfactory. This is of importance especially if the studies are focused on the brain, because the penetration of the BBB might be a rate limiting step.

Using PET in *in vivo* studies a rapid degradation of metenkephaline and

substance P in the blood was evident (66). In studies applying other complementary techniques to reveal the fate of the label, a rapid degradation in the blood was shown. Thus the brain radioactivity mainly was constituted of labelled metabolites. More stable enkephaline analogues have given evidence that at least partial BBB penetration of intact peptides may occur (66) (Figure 13; see color plate).

With increasing knowledge of the function and physiological role of neuropeptides, PET might be a powerful tool in the elucidation of their *in vivo* disposition after different routes of administration.

VI. CONCLUSIONS

The use of positron emission tomography as an *in vivo* technique to study the kinetics of suitable labelled tracer molecules of physiological or biological processes in the tissue of living animals or humans has opened new horizons to obtain increased knowledge in neuro-physiology and neuro-pharmacology. The use of several tracers in a series of experiment to visualize different processes of neurotransmission might in the future give us a powerful tool to study both the normal and the patho-physiological states of these processes. Several applications of PET in studies of neurotransmission and receptor function have thus already been applied clinically. In the future there are a lot of possible studies to be performed using PET which will have a large impact on our knowledge of neuronal function. We are also now seeing a beginning to how PET can be used as a valuable tool in the evaluation of clinical and experimental drugs.

The use of PET in multivariable analyses in multitracer studies of tissue kinetics and hence dynamics will further increase the power of the technique. We are only in the dawn of a new era which will improve our knowledge of brain function and hence improved treatment of neurological and psychiatric diseases.

However the success of PET in studies on neurotransmission and receptor binding can only be reached with a careful choice of tracer molecules, cautious interpretation of results, use of reliable models and careful assessment of other factors in PET-studies such as the determination of metabolites and changes in disposition of the tracer due to e.g. alterations in blood flow.

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7
Positron Emitter Labeled Enzyme Inhibitors and Substrates

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I. INTRODUCTION

Of the new medical imaging modalities, Positron Emission Tomography (PET) is uniquely capable of examining biochemical transformations and measuring drug pharmacokinetics in the living human and animal body (1). In essence, the PET method involves administering a positron emitter labeled radiotracer and measuring the accumulation of labeled product. The positron

emitted from a decaying isotope annihilates by interacting with an electron producing two body-penetrating photons which are emitted at an approximately 180 degree angle to each other. The detector (the positron emission tomograph) uses coincidence detection to locate the source of the annihilation photons within the object and to measure the concentration of the labeled compound. This is a technique which can be safely applied in humans because the commonly used isotopes have a short half life and decay to non-radioactive daughter products (Table 1). In addition, the specific activity of radiotracers labeled with the positron emitters is exceedingly high allowing their use as true tracers even for such pharmacologically sensitive measurements as receptor-radioligand interactions. Although the translation of the radioactivity concentration in tissue as measured by PET (and the radioactivity concentration in plasma, or in some reference tissue) to meaningful biochemical parameters (ie rate of glucose metabolism, concentration of neurotransmitter receptor, tissue pH, etc.) requires the application of a tracer kinetic model, it is important to emphasize that it is the design of highly selective radiotracers, the biochemical characterization of the uptake process, and the identification of the rate limiting reactions which are the foundation of the PET method.

Table 1. Physical Properties of ^{11}C, ^{15}O, ^{13}N and ^{18}F

NUCLIDE	HALF-LIFE	DECAY MODE	DECAY PRODUCT	MAXIMUM SPECIFIC ACTIVITY (Ci/mol)
^{11}C	20.4 min	β^+ (99+%)	^{11}B	9.22×10^9
^{15}O	2.07 min	β^+ (99.9%)	^{15}N	9.08×10^{10}
^{13}N	9.96 min	β^+ (100%)	^{13}C	1.89×10^{10}
^{18}F	109.7 min	β^+ (97%)	^{18}O	1.71×10^9

II. PROBING FUNCTIONAL ENZYME ACTIVITY WITH PET

Because of the importance of enzymes to virtually all biochemical transformations, the identification of abnormalities in enzyme concentration or reactivity and the modification of enzyme activity through the use of drugs holds the promise of understanding normal function and the progression and development of disease at the molecular level. Within the last decade, two basic approaches have been used for developing positron emitter labeled radiotracers which undergo metabolism to produce labeled products whose accumulation in tissue characterizes a simple biochemical transformation.

1. Metabolic trapping: This involves the use of a positron emitter labeled enzyme substrate which is converted to a labeled product which is trapped at the site of catalysis for the time period of the measurement (2).

2. Suicide inactivator approach: This involves the use of a positron emitter labeled suicide enzyme inactivator which undergoes a catalytically activated irreversible attachment to the enzyme resulting in the in vivo labeling of the enzyme (3).

It is noteworthy that metabolic trapping is the basis for the most widely used PET method, the 2-deoxy-2[^{18}F]fluoro-D-glucose (^{18}FDG) method for measuring regional glucose metabolism *in vivo* (4,5). Other tracers such as ^{13}N-ammonia also owe part of their uptake and sequestration to enzyme catalyzed incorporation into other molecules (6). One of the problems in relating the uptake of a radiotracer to the activity of a specific enzyme is that, with many of these tracers, the radiolabel cycles through many different enzymatic steps producing a variety of labeled products which may diffuse from the original site of catalysis. Additionally, the rate limiting step for uptake may not be the enzyme catalyzed step but may reflect some other factor such as blood flow.

This chapter will trace the progress in developing radiotracers for selectively probing two enzymes, hexokinase and monoamine oxidase and will

describe some of the general problems and pitfalls in developing positron emitter labeled radiotracers whose uptake *in vivo* reflects catalytically active enzyme.

A. Hexokinase

Hexokinase (ATP: D-hexose-6-phosphotransferase: E.C.2.7.1.1) catalyzes the first step in glycolysis, the conversion of glucose to glucose-6-phosphate. Mammalian hexokinases in various tissues occur in a multiplicity of types which are found both in the soluble and in the particulate fractions of homogenates. These enzymes form a control point for carbohydrate metabolism and the characteristics of the different hexokinases in different tissues reflects their particular role in that tissue. For example, brain hexokinase catalyzes the phosphorylation of glucose for the purpose of supplying energy to the tissue. Thus the activity of brain hexokinase depends on the demand for glucose-6-phosphate and tracks the metabolic activity of the tissue (7,8).

The key issue in measuring glucose metabolism with PET was the design and development of a radiotracer whose uptake could be related to one specific process, the hexokinase catalyzed phosphorylation of glucose.

Two approches have been used to measure glucose metabolism using PET. One approach is to use glucose labeled with the short-lived positron emitter carbon-11, and to make the measurement of radioactivity concentration at an early time after the injection of the tracer, before the labeled products diffuse from the original site of the hexokinase catalyzed phosphorylation and the labeled metabolites confuse the interpretation of the radioactivity measurement. A second approach uses a positron emitter labeled glucose analog which mimics the transport and metabolism of glucose but whose metabolism is blocked after the hexokinase catalyzed step producing a labeled product which is trapped within the cell for the time course of the PET

measurement. The use of each of these approaches is accompanied by tradeoffs which illustrate the complexity involved in probing a single biochemical transformation in a living system.

1. CARBON-11 LABELED GLUCOSE

Measurements of regional glucose metabolism have used uniformly labeled glucose produced by photosynthesis using $[^{11}C]O_2$ (9 and references therein). Since, in this case, the tracer ([^{11}C]glucose) is chemically identical to the molecule being traced (glucose) corrections for biological behavior are not required (10,11). However, since uniformly labeled glucose is rapidly converted to many labeled products (including $[^{11}C]O_2$) and these diffuse from the original site of hexokinase catalyzed phosphorylation, only the PET measurement of radioactivity concentration at early times reflects labeled glucose-6-phosphate and can be related to the hexokinase reaction. At early times, however, the observed tissue radioactivity must be corrected for a significant amount of radioactivity which is contained in the blood in the tissue for the first few minutes after injection.

Since the different carbon atoms of glucose have different metabolic fates, the selective labeling of glucose in a carbon atom which is more slowly converted to labeled products in the steps in the glycolytic cycle following hexokinase has been investigated as a means of more effectively trapping the label over the experimental measurement period for the measurement of regional cerebral glucose metabolism (12). It was found that [6-^{14}C]glucose and [1-^{14}C]glucose both undergo significantly less $[^{14}C]O_2$ loss than [U-^{14}C]glucose. For this reason, the use of both [1-^{11}C]glucose (which has been synthesized (13) and [6-^{11}C]glucose may be an effective strategy for extending the time period over which a meaningful PET measurement of radioactivity from the blood and improving counting statistics.

2. 2-DEOXY-D-GLUCOSE AND ITS DERIVATIVES

The use of positron emitter labeled 2-deoxy-D-glucose or 2-deoxy-2-fluoro-D-glucose is an extension of the [^{14}C]-2-deoxyglucose quantitative autoradiographic method for measuring regional brain glucose metabolism in animals (14). The design of these tracers grew out of the observations that the substitution of the hydroxyl group on C-2 of glucose does not interfere with the ability of the molecule to be transported into tissue or to serve as a substrate for hexokinase (15). The absence of a hydroxyl group on C-2 does, however, prevent the product of the hexokinase reaction (2-deoxy-D-glucose-6-phosphate or 2-deoxy-2-fluoro-D-glucose-6-phosphate) from undergoing the next step in the glycolytic cycle. Since the product of the hexokinase catalyzed phosphorylation is predominantly in the ionized form at physiological pH, it is intracellularly trapped at its site of formation. Thus the measurement of the regional distribution and concentration of 2-deoxyglucose-6-phosphate provides a map of the distribution and activity of the enzyme, provided that the time course of the tracer in the arterial plasma is known and that a number of other conditions are met (14).

The positron emitter labeled 2-deoxyglucose derivative, 2-deoxy-2-[^{18}F]fluoro-D-glucose (^{18}FDG) was the first positron emitter labeled 2-deoxyglucose derivative to be synthesized and used in the measurement of regional brain glucose metabolism in the living human brain with PET (5 and references therein). Later, carbon-11 labeled 2-deoxy-D-glucose was developed because the shorter half-life of carbon-11 relative to fluorine-18 (20.4 minutes vs. 110 minutes) is convenient for use in serial studies in the same individual at relatively short time intervals (16,17).

In spite of the fact that the labeled product spectrum is simpler with 2-deoxy-D-glucose and 2-deoxy-2-fluoro-D-glucose than with glucose itself, the measurement of regional brain glucose metabolism requires the use of a "lumped constant" to compensate for the fact that the tracer and the molecule

being traced are not identical. The "lumped constant" for [^{11}C]-2-deoxy-D-glucose and ^{18}FDG have been measured for normal brain, but may have a different value for diseased tissue (18). A method recently described for measuring the lumped constant on a regional basis in the living brain using PET and serial tracer studies with 3-O-[^{11}C]methylglucose and ^{18}FDG represents an approach to determining this important parameter in normal and diseased brain tissue (19).

The preceding sections serve to illustrate some of the complexities involved in developing radiotracers for probing a specific biochemical transformation using PET. Some of these same approaches have been applied to the development of positron emitter labeled tracers for probing monoamine oxidase with PET and are described in the following sections.

B. Monoamine Oxidase (MAO)

MAO (E.C. 1.4.3.4) is a flavin containing enzyme which is located on the outer mitochondrial membrane. It oxidatively deaminates amines from both endogenous and exogenous sources playing a key role in the regulation of the concentration of neurotransmitter amines. It exists in two forms, MAO A and MAO B, which are identified by their substrate selectivity and their sensitivity to different inhibitors (20). In general, MAO A resides within the neuron whereas MAO B is found outside of the neuron (21). When the substrate selectivity and the cellular compartmentation of the two forms of MAO are considered in light of their known different relative ratios in different organs, the potentially powerful role which they play in effecting subtle changes in the concentrations of neurotransmitter amines can be appreciated.

Interest and speculation on the significance of MAO in the regulation of the concentration of neurotransmitter amines was stimulated by the observation twenty-five years ago that the first drugs to exhibit antidepressant effects were MAO inhibitors (22). A later study suggested that individuals with low

platelet MAO demonstrated a vulnerability to psychiatric illness (23). Although the assay of platelet MAO and MAO concentration in post-mortem brain tissue has stimulated considerable speculation as to the role of MAO in a number of diseases, both of these methods have disadvantages when it comes to identifying a linkage between abnormalities in the enzyme and the development and progression of disease (24). Clearly, a nondestructive method for examining MAO in the living human brain would provide new insights.

The development of positron emitter labeled tracers for probing MAO activity *in vivo*, began over a decade ago with an investigation of the use of a series of carbon-11 labeled aliphatic amines of varying carbon chain length and radiorespirometry (25). It illustrates some of the problems in relating the production of labeled products to the activity of a single enzyme.

1. RADIORESPIROMETRY WITH CARBON-11 LABELED ALIPHATIC AMINES

The aliphatic amines are substrates for MAO undergoing the following reaction:

$$R(CH_2)n[^{11}C]H_2NH_2 \text{ --> } R(CH_2)n[^{11}C]HO + NH_3$$

However, the labeled product, the carbon-11 labeled aldehyde, is a substrate for the enzyme aldehyde dehydrogenase and is converted to the labeled carboxylic acid which undergoes beta oxidation to produce labeled carbon dioxide as the metabolic end-product.

$$R(CH_2)n[^{11}C]HO \text{ ---> } R(CH_2)n[^{11}C]O_2H \text{ ---> ---> } [^{11}C]O_2$$

The pivotal role played by MAO in the metabolism of the aliphatic amines such as carbon-11 labeled octylamine is illustrated by the influence of MAO inhibitors such as iproniazide on the production of labeled CO_2 by the mouse (25) (Figure 1).

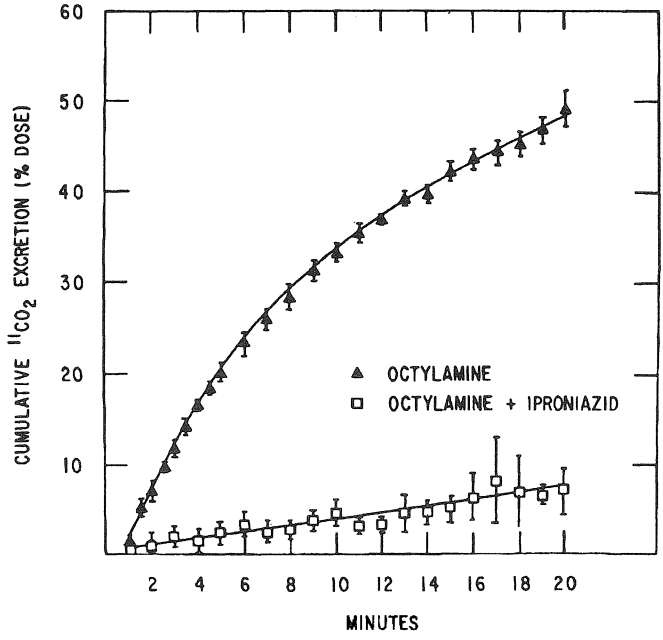


Figure 1. The Effect of Pretreatment with Iproniazid on the Cumulative $^{11}CO_2$ Excretion After Administration of ^{11}C-labeled Octylamine [25]

Here it can be seen that by inhibiting MAO, $[^{11}C]O_2$ production is nearly abolished, suggesting that the measurement of the production of labeled carbon dioxide after the administration of a carbon-11 labeled aliphatic amine might be a useful measurement of MAO activity. This was examined in more detail by another study in which MAO activity was inhibited by the irreversible MAO inhibitor, pargyline, and both labeled carbon dioxide production and the MAO activity of individual organs was measured at different times after the dose of pargyline (26). Measurements were made over the time period 1-96

hours during which MAO was synthesized to replace MAO which was irreversibly inhibited by pargyline. It was found that the return of the production of labeled carbon dioxide to control values roughly tracked the return of MAO activity by the intestine and to a lesser extent, the liver, but that brain MAO activity was only 20% of the control value when carbon dioxide production had recovered to 94% of the control value (Figures 2 and 3).

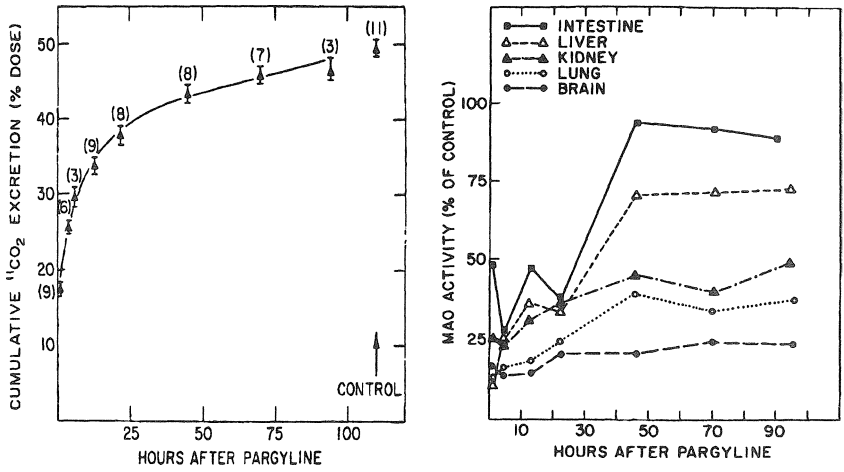


Figure 2. Cumulative $^{11}CO_2$ Excretion Expressed as Percent of Injected Dose excreted at 20 (▲) Min After the I.V. Injection of [^{11}C]octylamine at 1-94 Hr After Pargyline (10 mg/kg) Pretreatment. Control Value is Plotted at the Far Right for Comparison. The Number of Animals Used for Each Point is in Parentheses [26].

Figure 3. MAO Activity (for [^{14}C] octylamine) of Major Mouse Organs at 1-94 Hr After Pretreatment with Pargyline (10 mg/kg) [26]. Points Are The Average of Two Determinations. Percent of Control MAO Activity Represents:

$$100 \cdot \frac{\text{nmoles product mg of mitochondrial protein (pargyline-treated)}}{\text{nmoles product mg of mitochondrial protein (saline controls)}}$$

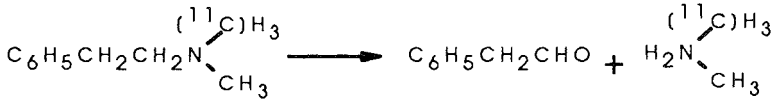
This study demonstrated that the turnover rate for MAO was different in

different organs and that the rate of carbon-11 labeled carbon dioxide production did not reflect MAO activity in organs such as brain and lung, which contain relatively low MAO concentrations. Thus although the measurement of $[^{11}C]O_2$ production represented an attractive initial approach to measuring MAO activity in some organs like brain, a more selective radiotracer, one which isolated the MAO reaction from other reactions and one which directly probed MAO activity in a specific organ was needed.

The following sections will summarize the progress to date in the development of specific positron emitter labeled radiotracers to map MAO.

2. THE LABELED SUBSTRATE APPROACH

N,N-Dimethylphenethylamine (DMPEA) is a good substrate for MAO B. When the positron emitter labeled substrate N-[^{11}C-methyl]N,N-dimethylphenethylamine [^{11}C] DMPEA is oxidized by MAO, carbon-11 labeled methylamine is produced.

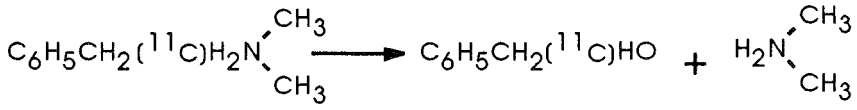


Since methylamine exists predominantly in the protonated form at physiological pH, it remains inside the cell where it is produced. This forms the basis for its use in the measurement of MAO activity in brain (27). Although the other product of the reaction, phenylacetaldehyde undergoes further metabolism, it (and its metabolites) is unlabeled and is therefore not detected. This can be contrasted to the behavior of the carbon-11 labeled aliphatic amines where the metabolically produced non-amine product bears the label.

Studies of carbon-14 labeled DMPEA in mice showed that labeled methylamine is indeed trapped in brain in control mice and that there was a

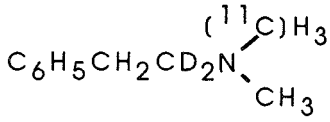
dose dependent decrease in dimethylamine production in animals who had received MAO inhibitors (27). This provided strong support for the contention that the measurement of radioactivity concentration with PET after the injection of [^{11}C-methyl]DMPEA would track MAO activity.

Subsequent studies of DMPEA labeled with carbon-11 in the alpha methylene group showed a completely different profile of radioactivity distribution with time (28). Because the products of MAO catalysis now consist of the labeled non-amine product and unlabeled dimethylamine,



metabolic trapping of a labeled product is not observed and the labeled phenylacetaldehyde and other labeled products clear from brain tissue. This has been demonstrated by comparing the time-radioactivity profile in baboon brain with PET for [N-^{11}C-methyl]DMPEA versus [^{11}C-alpha-methylene]DMPEA (28).

A third mechanistic study related to the use of labeled DMPEA to probe MAO with PET is the use of the deuterium isotope effect to probe the rate limiting chemical reaction involved in the ultimate accumulation of product in the tissue. In this study, tissue distribution measurements in mice showed that the rate of accumulation of radioactivity in brain is reduced after the injection of [^{11}C-methyl]DMPEA-α,α-d_2,



indicating that the production of labeled dimethylamine is related to the cleavage of the alpha C-H bond in the transition state (29) (Figure 4). This result is consistant with many observations that MAO catalyzed oxidative deamination is significantly reduced by the substitution of an α-hydrogen atom by deuterium (30 and references therein).

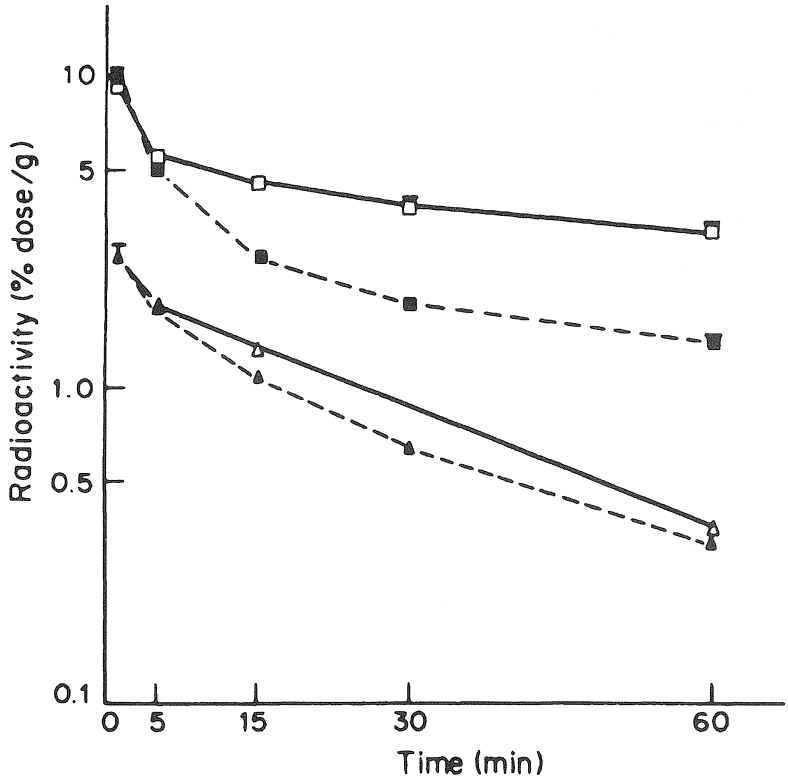


Figure 4. Time Courses of Radioactivity in the Blood and Brain After Injection of [$^{11}C_1$]DMPEA and [$^{11}C_1$]DMPEA-α,α-d_2 in Mice. (Δ–Δ) Radioactivity in Blood of [$^{11}C_1$]DMPEA; (□–□) Radioactivity in Brain of [$^{11}C_1$]DMPEA; (▲–▲) Radioactivity in Blood of [$^{11}C_1$]DMPEA-α,α-d_2; (■–■) Radioactivity in the Brain of [$^{11}C_1$]DMPEA α,α-d_2 (29).

3. LABELED SUICIDE ENZYME INACTIVATORS

The suicide enzyme inactivators (also called mechanism-based enzyme inhibitors) are enzyme substrates which contain a latent reactive functional group which is unmasked within the enzyme-substrate complex during the catalytic step. The catalytically activated substrate then forms a covalent bond to the enzyme causing its irreversible inactivation. This type of enzyme inhibition which depends on the catalytic activation of the substrate is commonly referred to as suicide inactivation because the enzyme catalyzes its own destruction (31).

A general kinetic scheme for the suicide inactivation process is given in Figure 5 (32). An important feature of suicide inactivation is that it is a branched pathway with normal enzyme turnover representing a competing branch. For this reason, the efficiency of suicide inactivation can vary depending on the relative rates of inactivation and turnover.

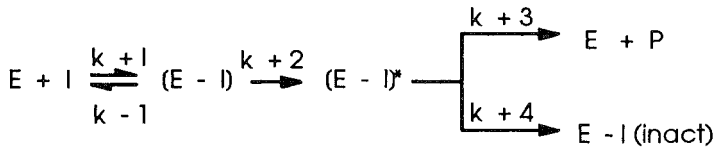


Figure 5. General Kinetic Scheme for the Suicide Inactivation Process (32), where E is the Enzyme, I is the Suicide Inactivator, [E-I] is the Enzyme-Substrate Complex, [E-I]* is the Catalytically Activated Suicide Inactivator Within the Enzyme-Substrate Complex, P is Product and E-I (Inact.) is the Inactivated Enzyme.

The use of positron-emitter labeled suicide inactivators offers the possibility of using the covalent bond formation between the labeled inhibitor and enzyme to selectively label the enzyme in vivo, providing that the following conditions are met:

1. The positron-emitter labeled inhibitor is transported into the organ of interest.

2. The positron-emitter labeled inhibitor is uniquely reactive with the

enzyme subtype (MAO A or B).

3. The suicide inactivation is rapid relative to the turnover of enzyme (see Figure 5).

The discovery and characterization of suicide inactivators for the two forms of MAO, MAO A and B, has made it possible to selectively examine their biochemical properties and represents one of the major breakthroughs in the study of MAO. These two compounds, clorgyline for MAO A (33), and L-deprenyl for MAO B (34), have been used as basic tools in the neurosciences (35) and also as investigational drugs in therapeutic applications to diseases involving abnormalities in monoamine concentration or metabolism (36). They, along with the antihypertensive drug, pargyline, have a common structural feature, the N-propargyl group (Figure 6). It is the propargyl group which is activated by enzymatic catalysis and it is through this group that the covalent bond to the reduced flavin cofactor of the enzyme is formed (37).

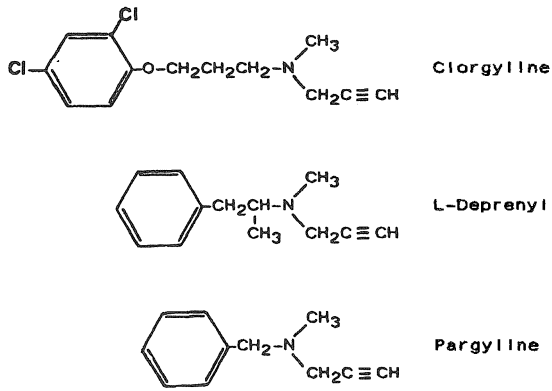


Figure 6. Structures of the Acetylenic MAO Inhibitors.

The feasibility of using carbon-11 labeled clorgyline and L-deprenyl to label MAO A and B *in vivo* and to map the regional concentration of MAO A and B in brain was initially investigated in mice (3). Animals were either

untreated (control) or pretreated with the unlabeled MAO inhibitors clorgyline and L-deprenyl in doses sufficient to inhibit MAO. Experiments were designed to probe whether the carbon-11 activity in different organs was covalently bound to protein, as one would expect if suicide inactivation and labeling of the enzyme had taken place. Pretreatment with the unlabeled MAO inhibitor clorgyline would be predicted to prevent covalent binding of labeled clorgyline but not labeled L-deprenyl to protein. Conversely, pretreatment with the unlabeled MAO B inhibitor, L-deprenyl, would be predicted to prevent covalent binding of labeled L-deprenyl but not labeled clorgyline to protein. Figure 7 shows the results of these studies for one organ, mouse brain, where it was found that the label did become attached to protein and the attachment could be prevented by pretreatment with the inhibitor for the same MAO subtype but not by the unlabeled inhibitor of the other MAO subtype.

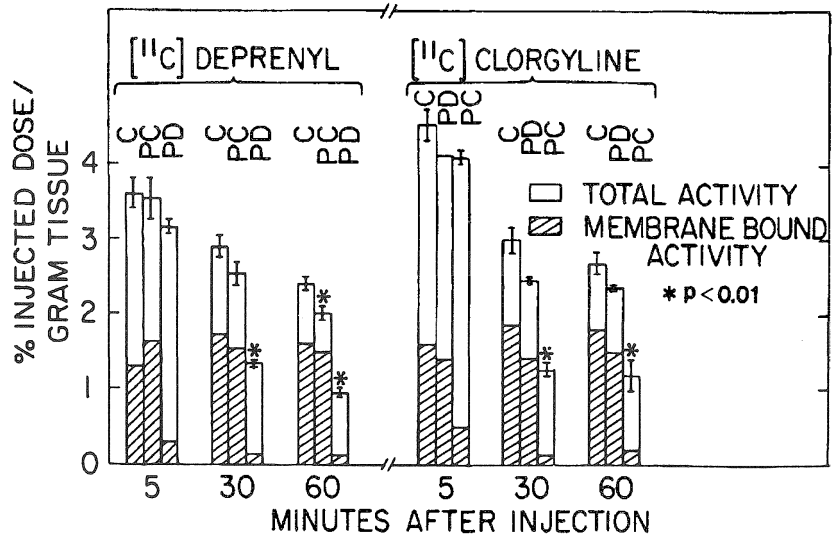


Figure 7. Time Course of Radioactivity in Brain of Control Mice (C) and Those Pretreated with Clorgyline (PC) or L-deprenyl (PD). Open Bars: Total Activity (Average ± S.E.M. of Four to Eight Animals). Hatched Bars: Activity Bound to Membranes (3).

The results of these studies and studies in baboons (38) supported an

investigation of the feasibility of using the carbon-11 labeled suicide inactivators to map MAO A and B in human brain (39). In four normal human volunteers, the temporal variation in radioactivity concentration in brain was measured over a 90 minute experimental time for carbon-11 labeled clorgyline and L-deprenyl. Both of these tracers were taken up and retained by brain tissue (Figure 8). For each subject, arterialized venous plasma samples were assayed for total radioactivity and for unmetabolized tracer.

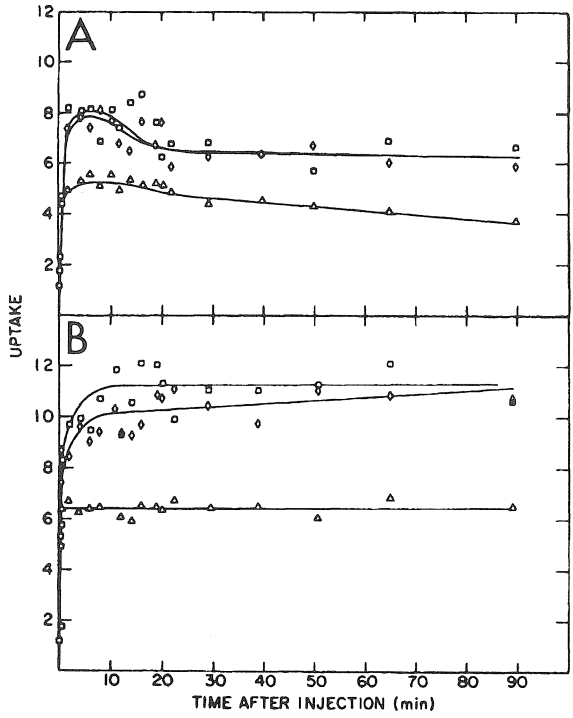


Figure 8. Time Course of Uptake for [^{11}C]clorgyline (A) and [^{11}C]L-deprenyl (B) in the Corpus Straitum (✧), Thalamus (□) and Cerebral Cortex (Δ) of a Normal Volunteer (age 86). Uptake Values Were Calculated From the nCi Per cm^3 Corrected for Decay for a Specific Brain Region Divided by the Radioactive Dose Given Per Gram of Body Weight (39).

The resulting arterial plasma input function and brain time-activity measurements from PET were used to calculate a blood to brain influx

constant (Ki, (40)) for each tracer. These serial studies were also carried out in one volunteer who was receiving the drug phenalzine (an inhibitor of MAO A and B) for the treatment of depression. A comparison of the blood to brain influx constants for the normal volunteers and the one subject who was treated with the MAO inhibitor, showed that MAO inhibition significantly reduced the value of the influx constant, suggesting that labeling of MAO was responsible for the observed uptake and retention of labeled clorgyline and L-deprenyl by human brain (39).

The labeled enantiomers of deprenyl were used to examine the retention of carbon-11 labeled L-deprenyl in more detail. Deprenyl contains an assymetric carbon atom and the D-deprenyl and L-deprenyl are synthesized from [S]-(+)-amphetamine and [R]-(-)-amphetamine, respectively. Recent studies *in vitro* in rat liver mitochondria showed that the MAO inhibitory potency of the L-enantiomer of deprenyl exceeds that of the D-enantiomer approximately twenty-five times (41). Since nonspecific distribution is generally not affected by stereoselective processes, experiments which compare the two enantiomers are useful for differentiating specific binding from non-specific distribution. In PET studies the initial uptake of the two enantiomers of deprenyl into human brain tissue was not significantly different. However, whereas [^{11}C]L-deprenyl was retained in MAO rich areas of the brain, the less active [^{11}C]D-deprenyl cleared rapidly. This rapid clearance of radioactivity after the injection of labeled D-deprenyl resembled that of labeled L-deprenyl in this same volunteer whose MAO was inhibited by a 15 mg dose of unlabeled L-deprenyl prior to injection of the labeled L-deprenyl (Figure 9). Thus stereoselective labeling of MAO B appears to be responsible for the long term retention of carbon-11 in brain tissue after the injection of labeled L-deprenyl.

In addition to the use of stereoselectivity as a mechanistic probe for assessing the involvement of MAO in the retention of L-deprenyl in brain, a

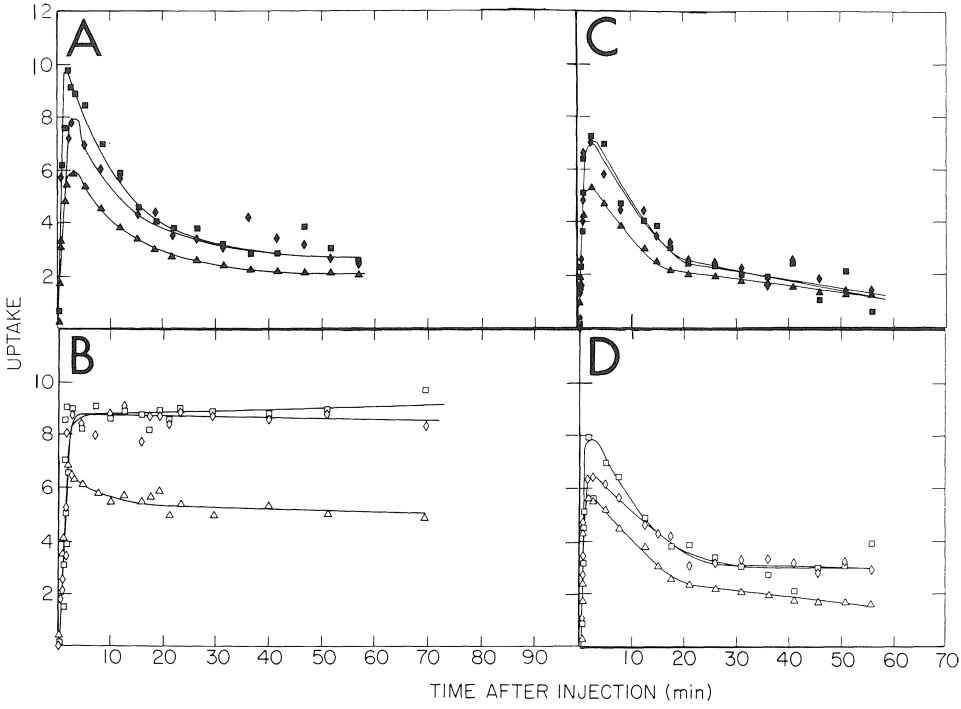


Figure 9. A Comparison of the Time Course of Uptake of [^{11}C]L-deprenyl (Open Symbols) and [^{11}C]D-deprenyl (Solid Symbols) in Striatum (✧,✦), Thalamus (❑,)

kinetic isotope effect has also been demonstrated (42). In this study, L-deprenyl, double-labeled (N-^{11}C-methyl and α,α,D_2(propargyl-methylene)) showed a significant decrease in brain retention in living baboon when compared to the parent ^{11}C-labeled L-deprenyl.

In vitro analysis of MAO activity in human autopsy material (43) and immunohistochemical analysis of MAO in non-human primate brain (44) have shown that both MAO A and B are present in human and primate brain. However, the ratio of MAO B to MAO A is approximately 4:1 (43). A number of studies have also shown that MAO is not uniformly distributed in the brain and that high concentrations occur in the thalamus, the basal ganglia and the hypothalamus (45). In addition, for MAO B, the frontal cortex MAO concentration is about two-fold lower than that of the thalamus (21). PET studies have demonstrated that both labeled clorgyline and L-deprenyl are retained in MAO rich regions of the human brain with the corpus straitum, the thalamus and the brain stem having the highest radioactivity concentration (Figure 10; see color plate).

Pargyline is another acetylenic MAO inhibitor. It does not selectively inhibit MAO A or MAO B but does have a preference for MAO B. It is also a less potent MAO inhibitor than L-deprenyl (46). The distribution of carbon-11 labeled pargyline was examined in mice to evaluate its potential as a positron emitter labeled tracer for MAO. Surprisingly, MAO did not appear to be responsible for the observed distribution of [^{11}C]pargyline in mice since pretreatment with unlabeled pargyline did not influence the biodistribution (47). Although [^{11}C]pargyline shows some specific retention in baboon brain, its influx constant is small relative to that of [^{11}C]L-deprenyl (38).

4. POTENTIAL APPLICATIONS OF PET STUDIES OF MAO

Although the characterization of the behavior of the positron-emitter labeled radiotracers for mapping MAO activity in human and animal brain is well underway, the application of this PET method to understanding the role which this enzyme plays in the development and progression of disease or in drug therapy is largely unexplored. From the studies which have been reported so far it appears that the use of either the labeled substrate approach with

[^{11}C]DMPEA or the labeled suicide inactivator approach (with [^{11}C]clorgyline and L-deprenyl) represent viable approaches to mapping MAI in the brain. Major differences in the two approaches are that DMPEA is predominantly an MAO B substrate limiting its use to the study of only MAO B while the suicide inactivators of both MAO A and B are available for selectively labeling each enzyme subtype. Additionally, the pharmacological profile of both clorgyline and L-deprenyl has been extensively characterized and these compounds have been used extensively as investigational drugs. Thus there is an extensive data base on their properties and their toxicology. The availability of these data has a significant impact on the ease with which application for human PET studies can be effected. Because both clorgyline and L-deprenyl are non-toxic at doses sufficient to inhibit significant amounts of the enzyme (36), it may be possible to use *in vivo* titration to determine MAO concentration directly in the living human brain as has been done previously with human autopsy material and to correlate these measurements with clinical state in individuals afflicted with a variety of pathologies. For example abnormal MAO activity has been implicated in variety of neurological and psychotic diseases and postmortem examination of human brain tissue or of platelet MAO has suggested that abnormalities in MAO may have important clinical consequences (48). Moreover, MAO B has been shown to play a key role in the conversion of MPTP to MPP^+, a neurotoxin which causes a Parkinson's-like syndrome in humans and monkeys (49).

The PET method for measuring MAO activity with carbon-11 labeled L-deprenyl has recently been used to measure the rate of recovery (synthesis) of MAO B after a single therapeutic dose of L-deprenyl (50) (Figure 11). This is a new approach to the measurement of the synthesis of a single protein *in vivo*, and a possible strategy for probing mitochondrial viability. The results of this study showed that MAO B synthesis in baboon brain is very slow with a half-life for recovery of about 4 weeks. Successful clinical trials of L-deprenyl

combined with L-DOPA for the treatment of Parkinson's disease (51,52) add additional relevance to methods which are capable of measuring its pharmacokinetics and determining other important clinical parameters such as the dose required to fully inhibit brain MAO and the time required for the resynthesis of MAO after treatment with irreversible MAO inhibitors.

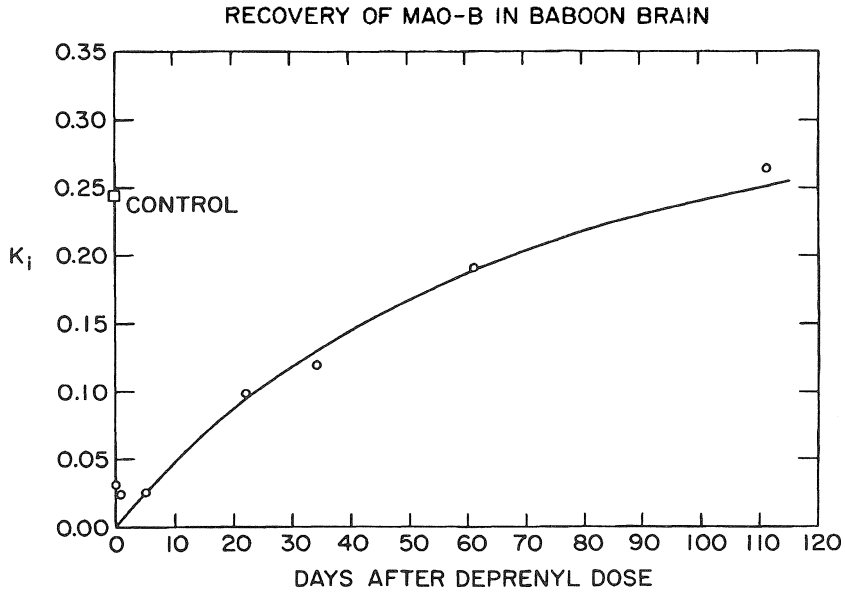


Figure 11. The Effect of a Single Intravenous Dose (1.0 mg/kg) of Unlabeled L-deprenyl on the Blood-To-Brain Influx Constants (K_i, in min^{-1}) for ^{11}C-L-deprenyl [50] in Baboon As Determined From Time Activity Data From Brain and Plasma.

III. POSITRON EMITTER LABELED TRACERS OF ENZYME ACTIVITY - THE FUTURE

Two fundamentally different approaches have been used to probe enzyme activity using PET. One of these, the use of a labeled substrate whose product is metabolically trapped, is the basis of the widely used ^{18}FDG method. The more recently described [^{11}C]DMPEA method for probing MAO is also based on the metabolic trapping within tissue of charged labeled product which does

not undergo other reactions within the time frame of the PET study. Enzymes which catalyze the synthesis of a product which bears a positive or negative charge at physiological pH are candidates providing that there is not significant egress of the uncharged form of the product from its site of synthesis over the time course of the study.

The second approach, that of using a suicide inactivator to label a single enzyme is an extension of a widely used strategy for selective enzyme inhibition. There are a large number of suicide inactivators which have been described in the literature (53). Some of these such as α-difluoromethylornithine, a suicide inactivator of ornithine decarboxylase, have been labeled with carbon-14 and used to obtain autoradiographic maps of enzyme activity in animals (54). It may indeed be possible to use some of these studies as guidance in developing selective radiotracers for PET.

One potential problem which must be kept in mind with the suicide inactivator approach is related to the general kinetic scheme for suicide inactivation shown in Figure 5. This is a branched pathway where the activated substrate in the enzyme-substrate complex [E-I]* can either react to form a product with turnover or release of the enzyme (E + P) or deactivate the enzyme by binding to it (E-I(inact)). It is the predominance of the latter pathway which is required if this approach is to be successful for labeling the enzyme in vivo. The ratio of product formation to suicide inactivation is called the partition ratio (32) and substrates with a high partition ratio are poor candidates as radiotracers for covalent labeling of enzymes in vivo.

In summary, PET is uniquely capable of providing insight into biochemical transformations in the living human and animal body. Although the majority of the investigations to date have addressed some aspect of brain biochemistry and abnormalities associated with neurological and psychiatric disease, the use of PET to map enzyme activity in other parts of the body can be expected to increase with the development of new radiotracers and the

increasing availability of high-resolution, whole-body positron emission tomographs.

Acknowledgement

This chapter was written at Brookhaven National Laboratory under contract DE-AC-76CH0016 with the U.S. Department of Energy and Supported by its Office of Health and Environmental Health. The author is grateful to Alfred P. Wolf for his advice and suggestions in preparing this manuscript.

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8

The Pharmacology of Steroid Radiopharmaceuticals: Specific and Non-Specific Binding and Uptake Selectivity

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I. INTRODUCTION

The purpose of an in vivo diagnostic radiopharmaceutical is to provide in a patient a distribution of radioactivity that can be imaged and then related adequately to physiological parameters, so that a clinically meaningful diagnostic decision can be reached. The required characteristics of the distribution will depend upon the nature of the clinical investigation.

While pharmacology has traditionally been a discipline concerned with therapeutic drugs, many parallels can be drawn between pharmacological considerations in the therapeutic and in the diagnostic realm: adsorption, distribution, potency, selectivity, duration of action, metabolism, excretion, etc. In each case, however, the issue may be somewhat different. For example, selectivity: With radiodiagnostic drugs, selectivity refers simply to the distribution of activity at target vs. non-target sites, since nuclear emission properties are not site dependent; by contrast, the selectivity of a therapeutic agent will depend both on its concentration at the desired vs. undesired sites and the particular biological responsiveness of each site. In both the diagnostic and therapeutic realms, however, the generation of metabolites creates new chemical species whose distribution may be important - with therapeutic drugs if the metabolites retain biological activity, and with radiodiagnostic agents if they retain the radiolabel.

It is the goal of the therapeutic pharmacologist to develop agents that will provide: (1) an adequate contrast between target and non-target areas, (2) a determinable relationship between the distribution of the activity and the distribution of relevant chemical species (3) an interpretable relationship between the distribution of chemical species and physiological parameters, and (4) an appropriate time course for the development and persistence of the activity distribution.

Efforts are underway to develop steroid radiopharmaceuticals as agents to evaluate receptor-positive tumors of the breast (estrogens and progestins),

ovary (estrogens) and prostate (androgens) in terms of prognosis for therapy, and to image steroid-hormone receptors in the brain. Up to now, most efforts have been focused on the estrogens, and while there are numerous reports of radiosyntheses, receptor binding and basic distribution studies, the overall picture of the pharmacology of steroid radiopharmaceuticals remains rather spotty.

It will be the scope of this review to present a conceptual overview of some of the factors that are important in developing steroid radiopharmaceuticals - those factors that relate to receptor and non-receptor binding affinity - that are critical to the development of high contrast between target and non-target areas. The issue of the rate and pattern of metabolism will not be addressed here, as it is reviewed elsewhere in this volume [R. N. Hanson chapter; this volume].

II. MODELS FOR IN VIVO BINDING DISTRIBUTION

Two general types of models have evolved in connection with the design and use of receptor binding radiopharmaceuticals - equilibrium models and dynamic models.

Equilibrium models have been used to ascertain the basic feasibility of achieving a selective distribution of radiopharmaceutical based on the concentration of receptor and in vitro ligand affinity for receptor and non-receptor binding sites. While perhaps overly simplistic in concept, these models have been very useful in guiding the basic design and structural optimization of radiopharmaceuticals in terms of target uptake potential and target to non-target tissue contrast. Dynamic models have been developed in order to relate the time course of target and non-target uptake and blood levels to relevant physiological parameters, such as receptor concentration and affinity, and certain tissue permeability rate constants. While the purpose of these two modelling approaches have, up to now, been distinct - design vs.

physiological evaluation - it is timely to consider merging these complementary perspectives.

A. Equilibrium Binding Models

1. BINDING INDEX OR RECEPTOR POTENTIAL MODEL

In the development of radioligands for both steroid and neurotransmitter receptors, Eckelman (1) evaluated potential ligands in terms of their binding affinity for receptor, more specifically, the ratio of the target tissue receptor concentration to the equilibrium dissociation constant of the ligand. This ratio (R_o/K_d) which elsewhere has variously been termed the "binding index" (2) or the "receptor potential" (3), should predict the bound to free ratio of tracer levels of ligand (low fractional saturation of receptor) under conditions of equilibrium.

Although useful in a comparative sense (i.e., enabling comparisons of different ligands and potential target sites in terms of their relative potential for bound to free contrast), this model is overly simplistic; it ignores the fact that the distribution is rarely an equilibrium phenomenon in vivo, since the preferential dissociation from low affinity sites and the clearance of free ligand affect the target to non-target contrast, and it ignores as well the contribution that non-receptor binding and metabolism may make to background (non-target) activity levels.

2. BINDING SELECTIVITY MODEL

The somewhat more advanced equilibrium model of Katzenellenbogen (4) considers non-receptor binding more explicitly. According to this approach, the binding selectivity of a compound, that is, its distribution between receptor and non-receptor binding sites, is considered to be the principal indicator of target tissue uptake selectivity in vivo. Certain justifiable simplifying assumptions enable this model to be used readily: receptor binding selectivity

is expressed by a binding selectivity index (BSI), which is the ratio of a compound's affinity for receptor vs. low affinity non-receptor (non-specific) binding. Both measurements of binding are normalized to that of a parent steroid; so, for example, with radiolabeled estrogens, the receptor binding affinity of estradiol is considered to be 100 and its non-specific binding to be 1, so that the standard compound has a BSI value of 100. Compounds with lower selectivities have BSI values below 100 and those with higher selectivity, above 100.

$$\text{Binding Selectivity Index} = \frac{\text{Receptor Binding Affinity}}{\text{Non-Specific Binding}}$$

$$\text{BSI} = \frac{\text{RBA (standard ligand = 100)}}{\text{NSB (standard ligand = 1)}}$$

This model has been used successfully in designing ligands for the estrogen receptor (see Section IV.A.), by relating quantitatively in vitro binding data to in vivo uptake selectivity, and it can be considered a simple strategy for the further perfection of the selective target site interaction and long duration of action of both diagnostic and therapeutic agents.

It is important to examine the simplifying assumptions in the binding selectivity index model: (1) The dynamic features of differential dissociation during clearance have been ignored; (2) even in the equilibrium sense, it is assumed that the background activity due to free ligand and labeled metabolites will be minimal, and (3) that binding to certain specific (non-receptor) binding systems, such as serum transport proteins, can be minimized by appropriate ligand design strategies (cf Section III.B.2.). In essence then, the degree to which this model makes valid predictions is a measure of the correctness of these assumptions (cf Section IV.A.).

B. Dynamic Models

In vivo studies with receptor-based radiopharmaceuticals for neuroreceptors in the brain and protein receptors in the liver have led to the evolution of pharmacokinetic models for target tissue uptake and retention (3, 5-7). It is the aim of these models to utilize the quantitative dynamic features of target tissue uptake - together with the dynamic profile of activity in non-target tissues and activity and metabolites in blood - to enable a valid estimate to be made of physiological parameters of importance: receptor concentration, binding affinity (or, in some cases, their product, the receptor potential (3)) and certain permeability limitations. These dynamic models have not yet been applied to the evaluation of steroid radiopharmaceutical uptake in target sites or tumors (see, for example, ref. 8), and they will not be discussed here in any detail. Nevertheless, these models have implications in the design of steroid radiopharmaceuticals, as one can learn from them and their applications in other systems certain key kinetic and metabolic features required for optimal behavior. In brief, these are the following: (1) The clearance of agent from non-target sites should be rapid with respect to the half-life of the radionuclide (so that adequate target to non-target contrast can be obtained); (2) circulating labeled metabolites should preferably be absent, or at least excluded from target and non-target areas where the correspondence between radioactivity levels and concentration of chemical species needs to be maintained (correction for circulating labeled metabolites in blood can be made), and (3) the binding parameters of the ligand for the receptor and non-specific sites should be adjusted such that uptake is not flow limited or such that substantial dissociation from the target site can occur during the lifetime of the radionuclide. (This enables receptor concentration or binding potential to be evaluated more definitively).

III. STEROID BINDING SITES

A. Receptor Concentrations in Target Tissues: Potential Uses in Diagnosis

Summarized in Table 1 are the concentrations of sex steroid hormone receptors in typical target tissues: The levels of estrogen receptors are highest in classical target sites such as the uterus; the levels in mammary tumors are generally lower, but cover a wide range, as some tumors are devoid of receptors while others may have levels higher than uterus. The levels in selected brain regions are yet lower, but consistently demonstrable by in vitro binding and autoradiographic methods. So appropriate estrogen radiopharmaceuticals might be used to provide functional images of breast tumors (see below), endometriosis, and brain receptors.

Progesterone receptors are found in uterine tissue and in some mammary tumors and in the brain (Table 1). As progesterone receptor is induced by estrogens, receptor levels may vary depending upon the endogenous levels of estrogens. This characteristic has been used to improve the correlation between breast tumor receptor positivity and responsiveness to estrogen hormonal therapy, since the presence of progesterone receptor indicates the presence of a functional estrogen receptor, thus distinguishing those tumors that may be positive for estrogen binding yet unresponsive to antiestrogen treatment (22,33).

In principle, images of receptor-positive breast tumors could be obtained with either estrogen or progesterone radiopharmaceuticals; these agents have somewhat different scope, however. The frequency of estrogen receptor positivity in breast cancer is somewhat higher than that of progesterone receptor, but as noted above, the estrogen receptor positive-progesterone receptor negative cases may have altered estrogen receptors that still bind ligand, but have lost the capacity to mediate estrogen stimulated responses (i.e., "non-functional receptors"). Still, the range of applicability of estrogen and progesterone agents in imaging primary and metastic tumors in patients

who have not undergone hormonal (antiestrogen) therapy is comparable. In contrast, patients on antiestrogen therapy have circulating levels of the antiestrogen and its metabolites that are sufficiently high to fully saturate the estrogen receptor (24,25). On the other hand progesterone receptors are not occupied in patients on antiestrogen therapy; their levels may actually be somewhat increased (25,26). Thus, only progestin-based agents could be utilized in patients on estrogen hormone therapy.

Androgen receptors are found in prostatic tissue and prostate tumors (Table 1). Significant levels are also found in some breast tumors. While the correlation between prostatic tumor androgen receptor levels and responsiveness to hormonal therapy is less evident than with estrogen and progestins in breast cancer, functional imaging of the prostate with an androgen radiopharmaceutical might prove useful in staging the cancer (27). Whereas the normal levels of circulating androgens are sufficiently high to saturate a significant fraction of the androgen receptors in the prostate (20), gonadal androgen production is reduced (28) in prostate cancer patients on estrogen therapy. Levels of free receptor are therefore elevated, and the tissue is more receptive to androgen radiopharmaceutical uptake (20).

Table 1. Sex Steroid Hormone Receptor Concentrations in Target Tissues

Receptor System/Target Site	Concentration (Ro; nM)	Kd (nM)	Receptor Potential (Ro/Kd)	Ref.[a]
Estrogen:		**(estradiol)**		
uterus (rat, immature)	30	0.2	150	--[b]
uterus (rat, mature diestrus)	10		50	9[b]
uterus (human, mid-proliferative)	3		15	10,11
mammary tumor (rat, DMBA induced)	3		15	12
breast tumor (human)[c]	0.3-3		1.5-15	13
pituitary (rat)	15		75	14,15
medial basal hypothalamus (rat)	1		5	16

Table 1 (Cont'd.)

Receptor System/Target Site	Concentration (Ro; nM)	Kd (nM)	Receptor Potential (Ro/Kd)	Ref.[a]
Progestin:		**(progesterone)**		
uterus (rat, immature estrogen-primed)	60	3	20	--[b]
uterus (rat, mature, proestrus)	7		2.3	17
uterus (human, late proliferative)	15		5	18
breast tumor (human)	0.3-3		0.1-1	13
pituitary (rat)	2		0.7	19
medial basal hypothalamus (rat)	1		0.3	19
Androgen:[d]	**(5α-dihydrotestosterone)**			
ventral prostate (rat, mature, 24h castrate)	5.7	3	1.9	20
prostate (human, normal)	0.5(2)		0.17	20
prostate (human, castrate)	0.6(1)		0.2	20
prostate (human, benign prostatic hypertrophy	0.3(2.9)		0.1	20
prostatic carcinoma (rat, Dunning, 24h castrate)	3.5		12	21
prostatic carcinoma (human, intact)	1.2(3.4)		0.4	20
(human, castrate)	0.7(1.7)		0.2	20
(estrogen therapy)	3.2(6.0)		1.1	20
(human, castrate and estrogen therapy)	3.1(3.2)		1.0	20
(mammary tumors (human)	0.5-1.5		0.2-0.5	13

a Calculated from the references noted. For conversion of receptor concentration data given only in p mol/mg soluble protein, a factor of 30 mg protein/g tissue was used.

b Our laboratory.

c Actual range is greater, but majority of "receptor positive" tumors lie between these values.

d Concentration data in parentheses are for total receptor, determined under exchange conditions. Only free receptor concentrations are used in the calculation of Ro/Kd.

If one considers a typical value for the equilibrium dissociation constant of the natural ligand for each receptor, it is clear from the equilibrium model of Eckelman (1) that adequate receptor potential (R_o/K_d) is available in the estrogen receptor system to generate a significant ratio of receptor-bound to free ligand. The R_o/K_d ratios for the progestin and androgen receptor systems

are lower, in large measure because the natural ligands for these systems, progesterone and dihydrotestosterone, have lower affinity. This suggests that higher affinity ligands will be of major importance in achieving adequate contrast and target tissue retention in these systems.

B. Binding Distribution of Steroid Hormones

1. HOMOLOGOUS RECEPTOR BINDING

Homologous receptor binding refers to the binding of a ligand to its intended receptor (e.g., estrogens with the estrogen receptor) and is contrasted with heterologous receptor binding (c., Section III.B.2.), which is ligand binding to other receptors (e.g., progestins binding to androgen receptor). It is beyond the scope of this review to cover in detail those structural features that lead to high affinity homologous receptor binding; good reviews on this topic are available (29-30). The structures of the natural ligands for the three sex steroid receptors, along with structures for some synthetic ligands, are shown in Table 2; other structures are given in Section IV. A number of points are worthy of comment.

The affinity of estradiol, the natural ligand for the estrogen receptor, is quite high. Affinity may be elevated somewhat more by inclusion of appropriate substituents on the 11β- and 17α-positions, and perhaps elsewhere, as well. Also, unlike the other two sex steroid receptors, the estrogen receptor has high affinity for a variety of synthetic non-steroid ligands, such as hexestrol. The simpler chemistry of these systems can be an advantage. Most antiestrogens share a triarylethylene structure, and some have high affinity for the receptor.

Progesterone, the natural ligand for the progestin receptor, has an affinity almost 10-fold lower than estradiol for the estrogen receptor. However, there are several synthetic progestin analogs both in the 17α-ethynyl-19-nortestosterone series and in the pregnane and retropregnane series that have

very much higher affinity. The recently developed antiprogestin, RU 486, is also a high affinity ligand.

Although testosterone is the principal circulating androgen, it is reduced by the 5α-reductase in target tissue cells to 5α-dihydrotestosterone (DHT), which has higher receptor binding affinity and is the major natural ligand for the androgen receptor. Like progesterone, the homologous binding affinity of DHT is considerably less than that of estradiol. Certain synthetic androgens have somewhat higher affinity for the androgen receptor than dihydrogestosterone. There are some steroidal and non-steroidal ligands for the androgen receptor that are antagonists, but their binding affinity is relatively modest.

Table 2. Structure and Homologous Receptor Binding of Natural and Synthetic Ligands for the Sex Steroid Receptors.

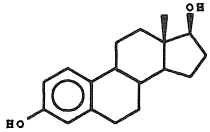
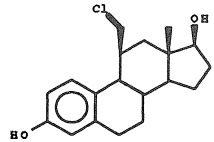
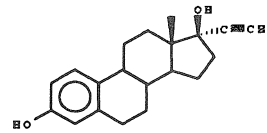
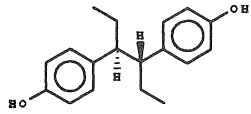
Natural Ligand (RBA)[a] [Kd,nM][b]	Synthetic Ligands (RBA)[a]
ESTROGEN	
 17β-estradiol (100) [0.3nM]	 ORG 4333 (1000-3000) 11β-chloromethylestradiol
	 ethynyl estradiol (140)
	 hexestrol (300)

Table 2 (cont'd.)

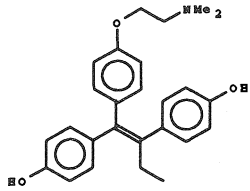
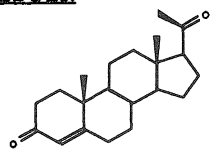
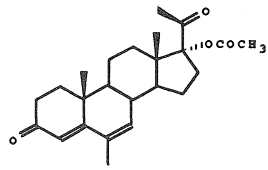
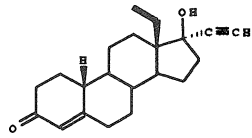
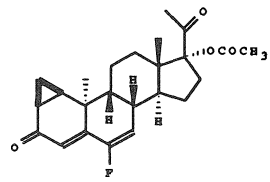
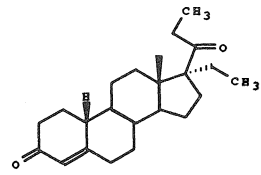
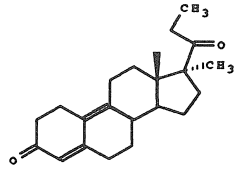
Natural Ligand (RBA)[a] [Kd,nM][b]	Synthetic Ligands (RBA)[a]
	 trans-hydroxytamoxifen antagonist (280)
PROGESTIN	
 Progesterone (100) [3 nM]	 chlormadinone acetate (120)[c]
	 norgestrel (170)[c]
	 DU 41,164 (>300)[d]
	 ORG 2058 (150)[c]

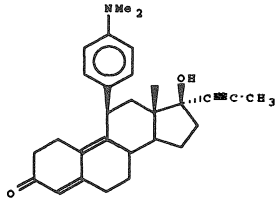
Table 2 (cont'd.)

Natural Ligand (RBA)[a] [Kd,nM][b]	Synthetic Ligands (RBA)[a]

PROGESTIN (cont'd.)

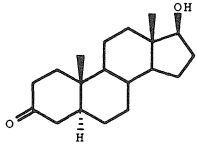


R 5020 (220)[e]
promegestone

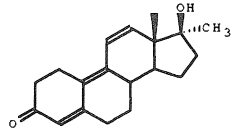


RU 486 antagonist
(530)[1]

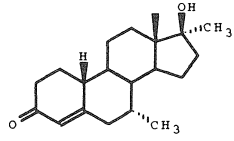
ANDROGEN



5α-dihydrotestosterone
(100) [1.4 nM][9]

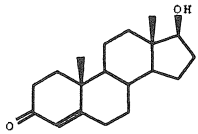
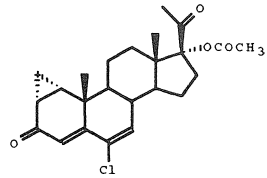
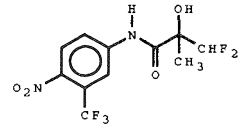


R1881, metribolone
(200)



mibolerone
(350)[h]

Table 2 (cont'd.)

Natural Ligand (RBA)[a] [Kd,nM][b]	Synthetic Ligands (RBA)[a]
ANDROGEN (cont'd)	
 testosterone (20)	 cyproterone acetate; antagonist (2)[i]
	 hydroxyflutamide derivative; antagonist (7)[i]

[a] RBA = Relative binding affinity (natural ligand = 100). Unless noted otherwise, data are from our laboratories.

[b] K_d for natural ligand.

[c] Ref. 29.

[d] Ref. 32.

[e] Ref. 30.

[f] Ref. 33.

[g] Ref. 34.

[h] Ref. 35.

[i] Ref. 36.

2. HETEROLOGOUS BINDING

While crossed or heterologous receptor binding is less of a problem with steroids than with other receptor systems such as neurotransmiters, it still is a matter of concern in radiopharmaceutical design. The estrogen receptor is very discriminating in that it binds with high affinity only substances that are

phenolic (30) (except in a few extreme cases (37,38)). In contrast, there is significant crossed specificity among the androgen, progestin, and glucorcorticoid receptors (30): progesterone and a number of synthetic pregnanes have significant affinity for the glucocorticoid receptor, and progestins of the nortestosterone class have significant affinity for the androgen receptor. There are, in fact, some ligands with high affinity for all three receptor systems (30).

Table 3. Generalizations Concerning Heterologous Binding of Steroid Hormones[a,b].

	Receptor System			
Ligand	**Estrogen**	**Androgen**	**Progestin**	**Glucocorticoid**
Estrogen	[HOMOL.]	(None)	(None)	(None)
Androgens	DHT and Δ^4-3β-ol, but (very weak)	[HOMOL.]	Δ^4,$\Delta^{4,9}$-,$\Delta^{4,9,11}$ 3-keto-estranes with 17α-Me or C≡CH (strong)	$\Delta^{4,9}$- & $\Delta^{4,9,11}$ 3-keto-estranes with 17α Me or C≡H (strong)
Progestin	(None)	19-nor-testo-sterones esp. $\Delta^{4,9}$, without large subst. at 16α and 17α (strong)	[HOMOL.]	$\Delta^{4,9}$- & $\Delta^{4,9,11}$ 3-keto-estranes
Glucocorticoid	(None)	(None)	(None)	[HOMOL.]

a This table is organized as a matrix, with ligands organized horizontally and receptors vertically. The diagonal elements represent homologous interactions (designated " [HOMOL]") and are not discussed. Where heterologous binding is not a substantial problem "(None)" is indicated.

b Summarized from Refs. 30,31.

These heterologous bindings have been studied in considerable detail. The generalizations presented in Table 3 provide a qualitative overview of this matter; more detailed reviews are available to enable the selection of ligands with high homologous and low heterologous binding (30,31).

3. HIGH AFFINITY NON-RECEPTOR BINDING

Steroid hormones are bound with high affinity by certain serum proteins (see Tabel 4). While these high affinity non-receptor serum binders present potential non-target sites of interaction that could lower the selectivity of target tissue uptake in vivo, the structural specificity of these binders is, in all cases, significantly different from those of the receptors (2,39). Thus, the binding of estrogens to alphafetoprotein in rodents or to sex steroid binding globulin in humans can be minimized by selecting non-steroidal ligands or by introducing substituents at the 17α- or 11β-positions. Similarly, the binding of certain synthetic progestins by corticosteroid binding globulin is minimal, whereas progesterone itself binds with high affinity.

There are several examples of diminished target tissue uptake selectivity due to high affinity non-receptor binding: the distribution of 16α-bromoestradiol in immature rats shows lower selectivity for estrogen target tissues because of binding by alphafetoprotein, which in rodents (but not in humans) has high affinity for certain steroidal estrogens (9). The binding selectivity is higher in older rats where alphafetoprotein levels are lower, and in young rats, high selectivity can be restored by utilizing the 11β-methoxy substituted analog which has high receptor affinity but low binding to alphafetoprotein (9). Obviously, then, a careful consideration of the species, age and pregnancy-dependence of high-affinity serum binder concentrations must be given in order not to be misled by the results from experimental animals in the search for steroid imaging agents to be used in humans.

Although its affinity for the estrogen receptor is quite high, the in vivo binding distribution of o-iodohexestrol is so low that target site uptake is not significantly different from non-target uptake (40). While part of the reduction in uptake selectivity is due to the high lipophilicity of this compound, its interaction with thyroid hormone binding proteins in serum may also be a factor. Trans-hydroxytamoxifen, which has very high affinity for the estrogen

receptor, shows less selective distribution in vivo than estradiol (41). This could be due to its high affinity for a microsomal binding site of wide distribution and unknown function (antiestrogen specific binding sites) (42).

Table 4. High Affinity Serum Binding Proteins for Steroids

Binding Protein	Plasma Source (Conc. nM)	Binding Specificity (Kd at °C, nM)
Sex Steroid Binding Protein (SBP, TEBG)	Human (non-pregnant 50-100nM; pregnant 200-400nM	Estradiol (2nM, 37°C) Testosterone (1.5nM, 37°C) DHT (1nM, 37°C) (Affinity reduced by 17α, 11β substituents; non-steroidal structure)
α-Fetoprotein (AFP)	Rat (immature, day 0 300μM; immature, day 21 1 μM; adult, 0.3nM)	Estradiol (25nM, 25°C) Estrone (13nM, 25°C) (Affinity reduced by 11β- and 17α-substituents; very low affinity for non-steroidal systems)
Corticosteroid Binding Globulin (CBG)	Human (non-pregnant, 600nM; pregnant, 1200nM)	Cortisol (25nM, 37°C) Progesterone (40nM, 37°C) (Affinity reduced by 9α-F, 16α-alkyl groups)

Summarized from Refs. 2, 39.

4. LOW AFFINITY, NON-SPECIFIC BINDING

Whereas one can "design away from" a high affinity non-receptor site whose binding profile is distinct from that of the receptor (cf. Table 4), certain low affinity binders have little structural specificity (2,39). Such binding, which probably represents the aggregate of hydrophobic surfaces on proteins and membrane and lipid phases, is much like a solvent partitioning, and standard measures of lipophilicity, such as octanol-water partition coefficients, can usually be used successfully to predict the magnitude of the binding (43,35). In fact, the calculated octanol-water partition coefficient formed the

basis for determining the binding selectivity index which has been used to predict in vivo uptake selectivity for estrogens based on their in vitro binding characteristics (4).

IV. IN VIVO UPTAKE OF STEROID RADIOPHARMACEUTICALS

Although there has been a longstanding interest in the preparation of steroid radiopharmaceuticals, it is only recently that attempts have been made to correlate structure, specific and non-specific binding in vitro, and uptake selectivity in vivo; many early studies involved inadequately characterized products resulting from the labeling of steroid ligands with radiohalogens, with little regard to receptor binding; the results, reviewed elsewhere (4), were generally of little significance.

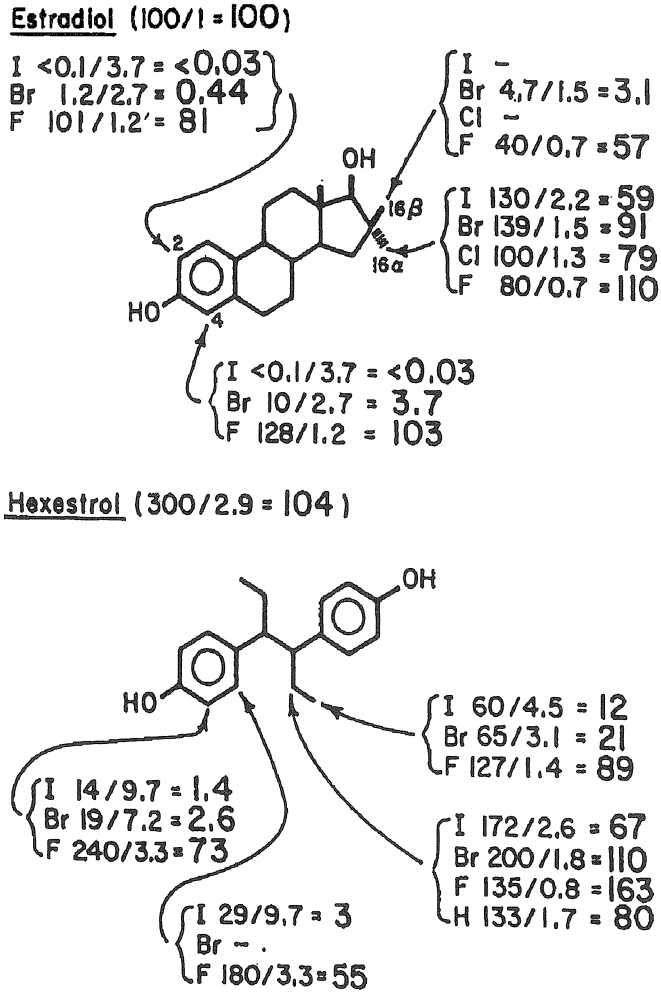
A. Estrogens

We have undertaken a series of studies (4,46-52) to investigate the relationship between the structure of halogen-substituted estrogens and their in vitro binding properties (to receptor and to non-specific binding proteins) and their in vivo uptake by target tissues. Shown in Scheme 1 is a pictorial representation of steroidal and non-steroidal estrogens, with an indication of the site of halogen substitution. In each case, the receptor binding affinity (relative to estradiol = 100) is shown, divided by the non-specific binding (relative to estradiol = 1), and in boldface the quotient, which is the binding selectivity index (BSI).

It is readily evident that the larger halogens (Br and I) are poorly tolerated in aromatic positions in either the steroidal or non-steroidal systems. They are tolerated at aliphatic positions (16α, but not 16β) on the steroid and on the hexane chain of hexestrol, either as a terminal substituent or in place of the methyl group. In contrast, fluorine, being only somewhat larger than hydrogen, is well tolerated at almost any position.

Scheme 1

Binding Selectivity Indices



To investigate the relationship between in vitro receptor and non-specific binding affinity, and in vivo target tissue uptake selectivity, six radiolabeled estrogens (both steroidal and non-steroidal), with affinities ranging from 60 to 240 with respect to estradiol (100) and non-specific binding ranging from 1 to 4.45 with respect to estradiol (1)) were injected into immature female rats, and

the ratio of activity in the uterus vs. four non-target tissues examined after 1 hour (Table 5, entries 1-6; Figure 1 (46,53)).

Table 5. Correlation Between In Vitro Receptor Binding Affinity, Binding Selectivity Index and In Vivo Target Tissue Uptake Selectivity[a]

Compound	RBA	NSB	BSI	Uterus/Non-Target[b] at 1 hr
1. estradiol (1)	100	1	100	13.3
2. 1-F-hexestrol (2)	130	1.4	93	11.4
3. 16α-Br-estradiol (3)	140	1.5	93	10.7
4. o-F-hexestrol (4)	240	3.3	73	9.5
5. 1-Br-hexestrol (5)	65	3.1	21	6.0
6. 1-I-hexestrol (6)	60	4.5	13	4.5
7. 11β-OMe-16α-estradiol (7)	20	0.17	118	19.8

[a]Summarized from refs. 44, 48, 49 and 51.

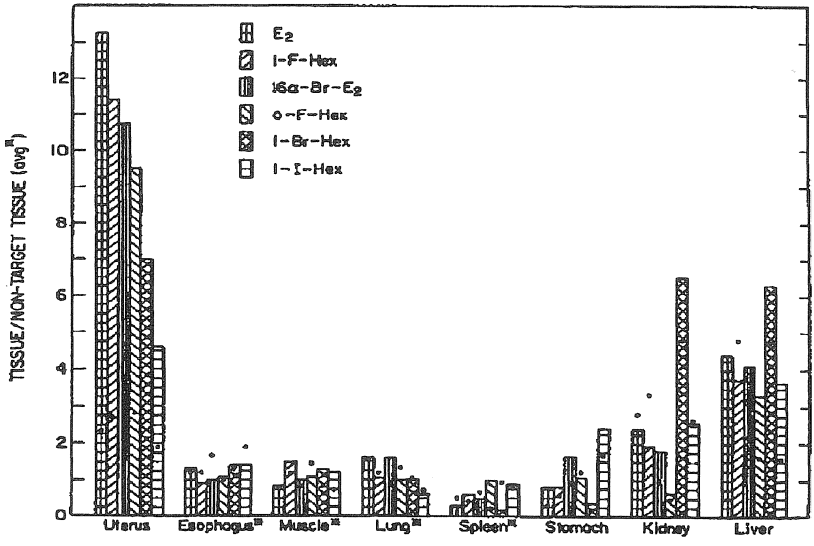


Figure 1. Tissue uptake selectivity of radiolabeled halogenated estrogen derivatives. Immature (day 25) female Holtzmann rats were injected intravenously (tail vein) with 2-20 μCi of the indicated compounds in the absence (vertical bars) or presence (dots) of an excess (13 μg) of unlabeled estradiol (to determine nonspecific uptake). Uptake levels, determined after 1 hour, are expressed as the uptake per gm relative to the average uptake in the nontarget tissues: esophagus, muscle, lung, spleen (from ref. 53).

The correlation between in vivo uptake selectivity and the binding selectivity index was gratifying and was far superior to the correlation simply with receptor binding affinity (that is, neglecting non-specific binding (Figure 2, (53)). While the quantitative success of this experiment may have been fortuitous, since only a single time point was investigated and the extent of metabolites in the target and non-target sites was not examined, it served to legitimize consideration of both specific and non-specific binding in radiopharmaceutical design.

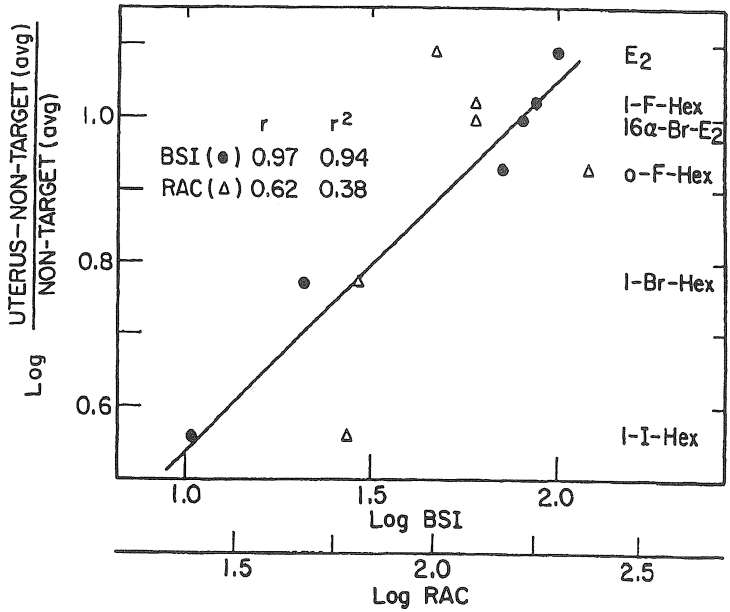


Figure 2. Correlation between target tissue uptake selectivity index and binding selectivity index and receptor affinity. The tissue uptake data (Figure 1) were recalculated in terms of the target tissue uptake selectivity (see text). The $\log_{10}$ of these indices were correlated with Me $\log_{10}$ of the binding selectivity indices (BSI) and the receptor affinities (RAC) of the individual compounds. The scales on the abscissa were adjusted so that the two correlation lines coincide. The structures of the compounds are evident from the abbreviations (from Ref. 53 and from Table 5).

More extensive studies have been performed on selected radiohalogenated estrogens: 16α-iodoestradiol, the first gamma-emitting estrogen to show selective uptake by target tissues in vivo, was prepared by Hochberg (54-57); more recently, the 11β-methoxy analog, which shows higher binding selectivity (due to lowered lipophilicity and reduced specific serum binding), has been shown to have more selective uptake in vivo (58,59). A similar improvement in uptake selectivity had been noted earlier in the 16α-bromoestradiol series (50,51): even though the receptor binding affinity of 11β-methoxy bromoestradiol is less than that of bromoestradiol, its non-specific binding is much lower, so that the binding selectivity (receptor to non-specific binding ratio) is higher (cf. Table 5, entry 7 vs. entry 3); the in vivo uptake selectivity correlates with the binding selectivities, not the receptor affinities. The in vivo uptake selectivity of 17α-iodovinyl estrogens has been studied in considerable detail. Again, uptake selectivity is improved by the introduction of the 11β-methoxy substituent, and very high uterus to non-target tissue uptake ratios are observed (60-64).

Iodine and bromine-substituted pentestrol derivatives (norhexestrol) have high affinity for the estrogen receptor, as they are isosteric with the high affinity non-steroidal estrogen hexestrol; their uptake selectivity was also quite high (65). The distribution of these compounds may be compromised to some degree by their solvolytic lability 65).

17α-methylestradiol, 17α-ethynylestradiol, and moxestrol (the 11β-methoxy analog of ethylnylestradiol) have been synthesized in C-11 labeled form, albeit in relatively low specific activity (6-90 Ci/mmol). At tracer doses, these compounds showed selective uptake in rat uterus and DMBA-induced mammary tumors (66,67).

We have prepared four fluorine-18 substituted estrogens - 16α- and 16β-fluoroestradiol, and fluoropentestrol and fluorohexestrol - and compared their in vitro and in vivo binding properties (52). The in vivo uptake selectivity of

all four compounds was very high, but 16α-fluoroestradiol appeared to be somewhat better than the other three (Figure 3). This compound can be prepared by a convenient two-step procedure (68) that has been adapted to robotic production (69), and encouraging positron tomographic images of human breast tumors and involved axillary lymph nodes have been obtained (70). Some of this work has been reviewed recently (71).

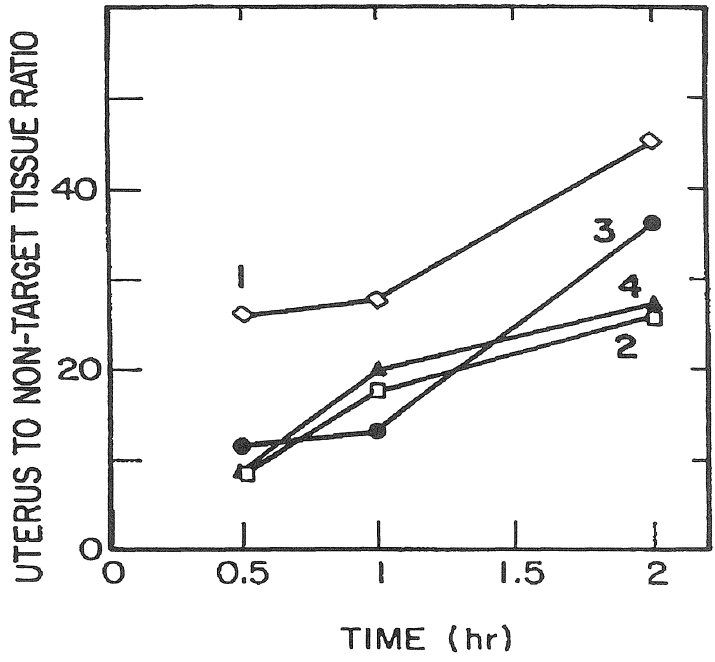


Figure 3. Uptake selectivity of four F-18 labeled estrogens in immature rats, expressed as uterus-to-nontarget tissue ratios (adapted from Ref. 52). Compound numbers are: 1, 16α[^{18}F]-fluoroestradiol; 2, 16β[^{18}F]-fluoroestradiol; 3, 1[^{18}F]-fluoropentestrol; 4, 1[^{18}F]-fluorohexestrol.

B. Progestins

To date, only limited studies on progestin radiopharmaceuticals have been undertaken. A number of earlier investigations to produce radioiodine-bromine-, or fluorine-labeled compounds gave material with either low

receptor affinity or limited metabolic stability. These studies have been reviewed eleswhere (4,31).

Recently, Hochberg (72,73) and Grill (74) have described that 17α-iodovinyl-19-nortestosterone binds to the progesterone receptor with high affinity. No in vivo uptake studies on this interesting compound have been described.

We have recently described a systematic investigation of fluorine-substitution on progestins in the testosterone and 19-nortestosterone series (75). We have found that fluorine substitution is well tolerated at the 16α and 11B positions; in addition, certain 16α-fluoropropyl, 17α-fluoromethyl, and 17α-(3-fluoro-1-propynyl) derivatives have affinities comparable to that of progesterone. To date, only the uptake selectivity of 17α-fluoropropynyl nortestosterone has been investigated, and it shows little target tissue selectivity, either because its binding affinity or binding selectivity is too low, or because it is metabolically unstable.

We have recently reviewed in detail structural factors that affect receptor binding affinity and biological activity of progestins in the pregnane, androstane and estrane series (31). In addition, there are a number of synthetic ligand systems that demonstrate even higher affinity for the progesterone receptor system (cf. Table 2). In fact, a fluorine-substituted analog of ORG 2058, 21-fluoro-16α-ethyl-19-norprogesterone, shows highly selective uptake into estrogen-primed rat uterus (76) and may well prove to be an effective imaging agent for progesterone receptors.

C. Androgens

Investigations on androgen radiopharmaceuticals have been even more limited. Again, earlier studies, reviewed elsewhere, generally describe compounds that were unstable, prepared only at low specific activity, or had poor receptor binding affinity (4,30,77).

We have made a systematic study of fluorine substitution in the testosterone and 19-nortestosterone series (74). Fluorine substitution at the 16α- and 11β-positions is well tolerated; fluorine-containing alkyl groups at the 16α- and 17α-positions, however, descrease binding. In vivo uptake studies with these compounds have not yet been reported.

Recently, we have reviewed in detail the effects of structural variation in the androstane and estrane series on androgen receptor binding affinity and biological activity (31). There are also a number of other high affinity ligand systems that could be considered for radiopharmaceutical design (Table 2).

V. CONCLUSION

The development of steroid hormone radiopharmaceuticals is a process that can be undertaken in a systematic and relatively rational fashion: the concentration of receptor sites in target areas are well defined, as are the potential non-receptor sites of interaction; studies relating structural changes to alterations in biological activity and receptor binding have been extensive because of interest in the pharmaceutical industry to develop effective and selective therapeutic agents as well as specific ligands for in vitro clinical assays of receptors.

This background of information, together with judicious considerations of radiolabeling strategy and potential effects of structural changes on the patterns of metabolism, enables one to approach the development of suitable imaging agents with some sense of directedness. This information can also be a guide to efforts to perfect the pharmacokinetic properties of these agents so that their time-dependent distribution can be analyzed by models that will provide appropriate quantitative information on receptor concentrations and binding affinity.

The major effort to date has been made with the estrogens, and several agents with very good in vivo uptake selectivity have been described, one of

which has been used to obtain impressive images of breast tumors. Investigations in the progestin and androgen area have been much more limited to date, but opportunities for success in these areas are no less.

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The Influence of Structure Modification on the Metabolic Transformations of Radiolabeled Estrogen Derivatives

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I. INTRODUCTION

A. Radiolabeled Steroid Derivative

The potential of radiolabeled derivatives of the estrogenic steroid hormones as tumor imaging agents can be associated with the discovery of the

uptake of [H-3] estrogen in breast carcinoma (1). The availability of high specific activity [H-3] estradiol subsequently permitted the identification of the mechanism of localization of estrogens which is now accepted as utilizing a specific intracellular hormone receptor in responsive tissues including estrogen responsive carcinoma (2,3). Numerous studies have subsequently linked the level of these estrogen receptors (as well as progesterone receptors) in the neoplastic tissue with the patient's suitability for a specific therapeutic regimen and overall prognosis (4-8). In general, the higher the receptor level the greater the likelihood of a favorable response to hormonal therapy. Because H-3 is a beta-emitter, a quantitative assay of the receptor levels requires removal by biopsy of a portion of the tissue in question for in vitro analysis. A more desirable method for characterizing the tissue in situ would be in vivo imaging using an appropriately labeled single-photon or positron-emitting steroid hormone. Such an agent would possess the advantages of being noninvasive, permitting repeat evaluations of the same site and being able, perhaps, to detect microscopic foci associated with early primary or metastatic disease.

As a result, the objective of radiopharmaceutical chemistry in the area of steroids over the past decade has focused on the synthetic and general biological properties of radiolabeled derivatives. There have been a number of reviews that have specifically discussed the rationale for the design and synthesis of both single photon and positron emitting estrogens (9-15). Substantial progress has been made, not only with respect to the development of agents that retain high receptor affinity and selectivity in vitro, but also with methods which allow them to be prepared rapidly and cleanly within the limits set by the radionuclidic properties (16-45). In several cases, for example, the [F-18] fluoroestrogens (38) and the [I-125, 123] iodovinyl estrogens (28-34), in vivo selectivity for the responsive tissue has also been demonstrated. As this area of research moves toward the next phase of development, with ultimate

clinical application in mind, processes within the organism that proceed concurrently with the target tissue localization must be considered (46). Of these, perhaps the most important processes for the steroid hormones - are the metabolic transformations. Phase I processes, which include oxidation, reduction and hydrolysis and Phase II processes, which include various conjugation reactions, will affect the levels of circulating labeled hormone and its metabolites, and their clearance from the plasma via hepatobiliary and renal excretion routes.

The discussion which follows is intended as a guide for those considering the structural modifications that may be employed in the design of radiolabeled estrogens (and presumably other steroid hormones and antihormones as well). A discussion of the effect of specific group substitution, e.g., the halogens (F, Br, I) or alkyl moieties, alkenyl or alkynyl upon specific receptor binding versus nonspecific receptor binding has been extensively described elsewhere and will be referred to only as it relates to the structures of potential agents of clinical interest. Instead, the remainder of the chapter will concentrate on the metabolic transformations of the major estrogens in mammalian species, including man and then attempt to identify the effects that the incorporation of specific radionuclide containing groups may have upon those pathways. Although many labeled estrogens have been reported, those which appear to retain the best receptor binding properties are limited to substitution on the A or D rings. Therefore, the classes of estrogens that will be specifically treated are those bearing (a) 1-2-/4-halo substitution, (b) 16-halo substitution and (c) 17, alkyl, alkynyl or haloalkenyl substitution. Other aspects of estrogen radiopharmacology are described in the chapter by Katzenellenbogen.

B. Primary Pathways for the Metabolism of Estrogens

Substantial literature exists that describes the metabolism of estradiol and its derivatives (47-51). In evaluating the published data and in making

estensions to the radiopharmaceutical chemistry of labeled estrogens several precautions must be made. One must be aware that most of the data were generated using [H-3] or [C-14] labeled materials and that these compounds were used at various specific activities. The route of administration, oral or intravenous, and the administered dose also affect metabolism, particularly when compared to nuclear medicinal applications which involve the intravenous administration of tracer doses. Lastly, the metabolic studies were performed in a variety of species such as the rat, dog, pig, monkey and man, and often resulted in marked species differences in pharmacokinetics and metabolic profiles.

Given these concerns, however, the metabolic pathways of several estrogens which are of interest to radiopharmaceutical scientists are available. The ones which have been chosen to exemplify the multitude of transformations that can occur are (a) estradiol 1; (b) ethynylestradiol 2; (c) mestranol 3 and (d) moxestrol 4 (Figure 1).

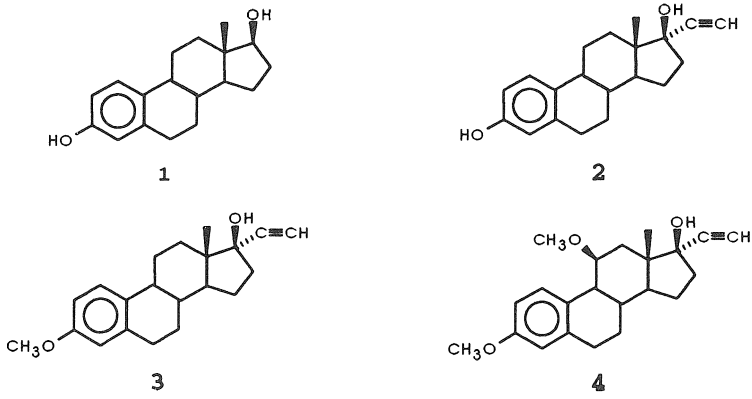


Figure 1. Structures of the major estrogens: estradiol 1, 17α-ethynylestradiol 2, mestranol 3, and moxestrol 4.

This small series will provide information regarding the Phase I and Phase II reactions that occur and the effect of the 17-substitution, of modification of A-

ring and 11-substitution, which are some of the structural modifications found or anticipated in radiopharmaceutical development.

1. ESTRADIOL 1

An abbreviated scheme for the Phase I metabolism of estradiol itself is shown in Figure 2.

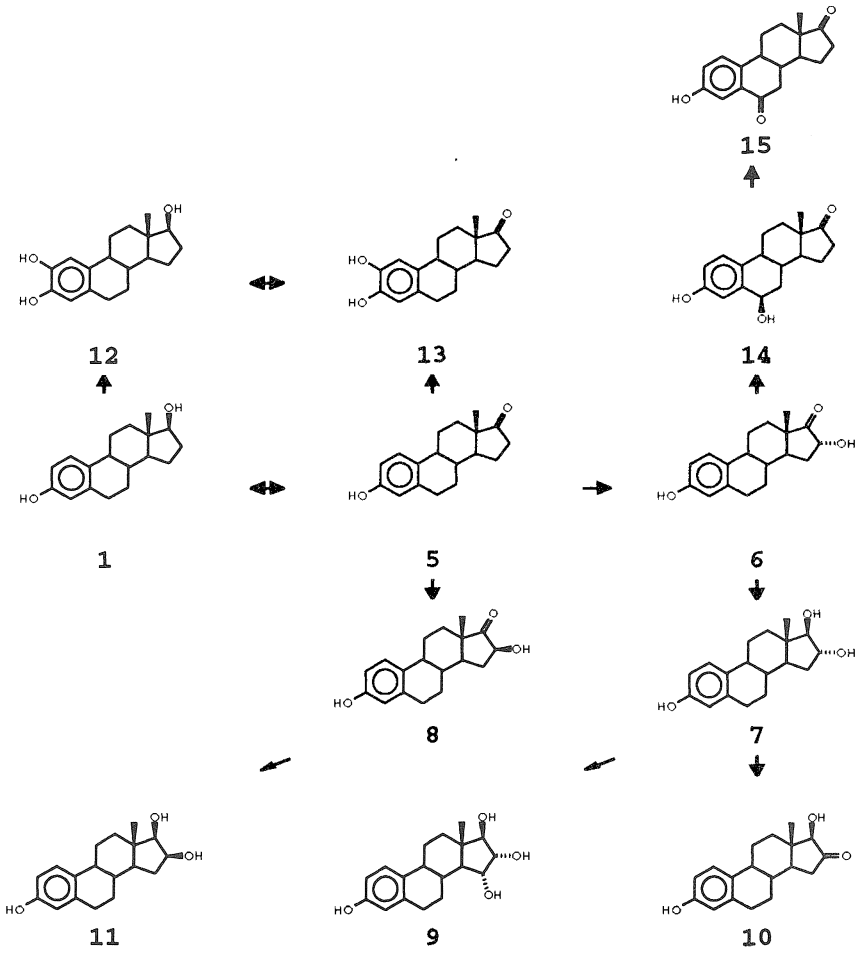
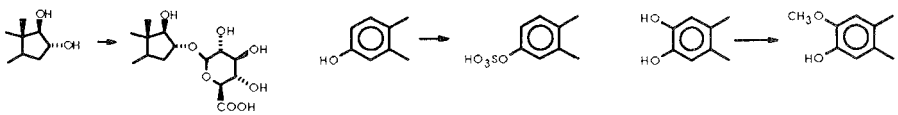


Figure 2. Brief summary of phase I metabolites of estradiol 1, including estrone 5, estriol 7, and catechol estradiol 12.

As can be seen, the major transformations involve oxidations and/or reductions on the A and D rings of the steroid (47). Although the conversion of estradiol to estrone is reversible and rapid, estrone is also an excellent substrate for several oxidative enzymes. As a result, oxidation occurs at the 16α- or 16β-positions, which, after reduction of the 17-ketone, yields the estriols 7 and 11. Further oxidation on the D-ring has been reported to yield the 16-oxo and 15α-hydroxy estrogens as well (49). Phase I metabolism of the A-ring occurs primarily at the 2-position to give catechol estrogens (52,53). Hydroxylations at 6- and 11- positions have also been observed (50), however, these are relatively minor in comparison to the reactions previously noted. Certainly, as will be discussed later, the introduction of substituents at or adacent to the major sites of oxidation will influence the metabolic profiles observed.

There are three major conjugative or Phase II reactions that occur with the estrogens and these are depicted in Figure 3. They are (a) glucuronidation, which occurs primarily on the D-ring hydroxyls, more frequently at the 16α- than the 17β-position; (b) sulfation, which involves the phenolic hydroxyl, and (c) methylation which is observed only with the catechol estrogens. To some degree, conjugation on both the A- and D-rings may occur within the same molecule.



(a) (b) (c)

Figure 3. The three major phase II pathways of estrogens; (a) - glucuronidation, (b) - sulfation, and (c) - O-methylation.

The transformations described in the preceeding section have important implications for the labeled derivatives. This can be depicted as in Figure 4.

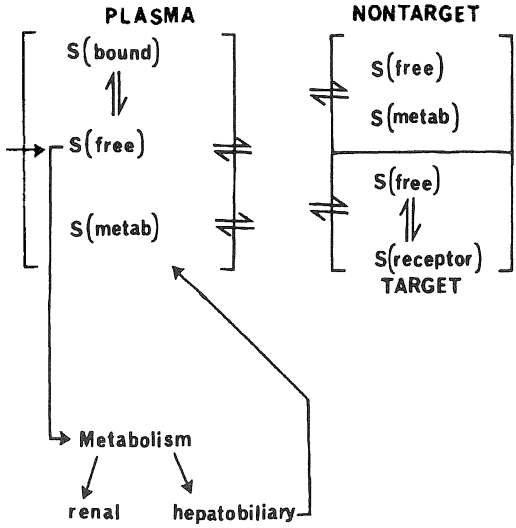


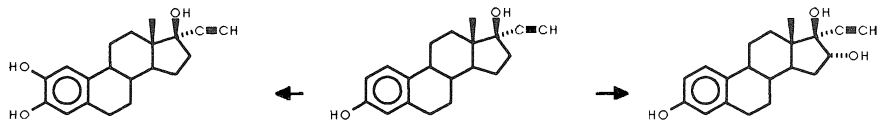
Figure 4. Brief summary of the disposition of estrogens in plasma and tissues following intravenous administration.

Most of the administered compound is distributed initially to all tissues (including nontarget sites) and then equilibrates between those tissues and the plasma. The rate at which the compound is deactivated, converted to water soluble conjugates and excreted by hepatobiliary/renal routes then will affect the target tissue uptake, as well as target to nontarget and target tissue to blood ratios. The nonconjugated metabolites such as estriols 7 and 11 can still permeate the tissues, including estrogen responsive tissues, but will not be bound significantly to receptors. The conjugated estrogen metabolites will be excreted initially about 50% by the hepatobiliary system and 50% by the kidneys (47). However, about half of the metabolites excreted into the gastrointestinal tract are reabsorbed via enterohepatic recirculation such that by 48 hours, over 75% of the metabolites are cleared through the kidneys. While

these rates may not affect short-lived radiopharmaceuticals labeled with C-11 or F-18, those labeled with Br-77 or I-123 would require considerations of the affects of these pathways, especially with respect to dosimetry.

2. 17a-ETHYNYLESTRADIOL 2

Structurally, the only difference between this compound and estradiol is the presence of the 17α-ethynyl moiety, yet it has a powerful influence on the disposition of the compound in vivo. It is clear from the following metabolic pathways (Figure 5) that oxidation on the D-ring is largely suppressed because oxidation of the 17β-hydroxyl to the corresponding ketone is not possible (47,50,54,57).



16 2 17

Figure 5. Primary phase I transformations of 17α-ethynylestradiol 2.

Therefore, the major Phase I metabolism occurs on the A-ring in the form of the 2-hydroxy product. Even this is not as great as the D-ring oxidations observed with estradiol. As a result, 17α-ethynyl estradiol exhibits a much slower clearance from the body with only 15-25% of the drug eliminated in the urine and 24-45% in the bile, over 72 hours, most of which is glucuronides and sulfates of the unchanged estradiol (57).

Another feature associated with the 17α-ethynyl estrogens is the observation that small amounts of the drug became irreversibly bound in vivo (58,59). It has been suggested that one reactive species is generated via oxidation of the 2-hydroxy metabolite. Evidence suggests that this

intermediate is an ortho-semiquinone that can readily alkylate proteins (59,60) (Figure 6).

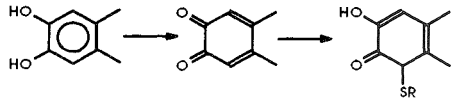


Figure 6. Transformation of 2-hydroxymetabolite to adduct with endogenous thiols.

The studies by Maggs, et al (60), indicate that this stems from the reactions with cysteinyl sulfhydryl groups. Another site where metabolic oxidation by the mixed function oxidases may lead to irreversible protein binding is the ethynyl group. The oxidation of the ethynyl moiety was shown by Ortiz de Montellano, et al (61) to lead to an active intermediate capable of binding to the heme group of the cytochrome P-450 enzyme (Figure 7).

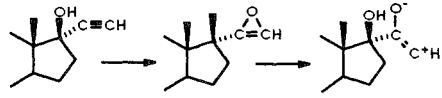


Figure 7. Activation of ethynyl group to a potent alkylating species.

3. 17α-ETHYNYLESTRADIOL-3-METHYL ETHER [MESTRANOL] 3

In general, the metabolism of the 3-0-methyl ether is similar to that of the parent compound, 17α-ethynyl estradiol (50,62,63) (Figure 8).

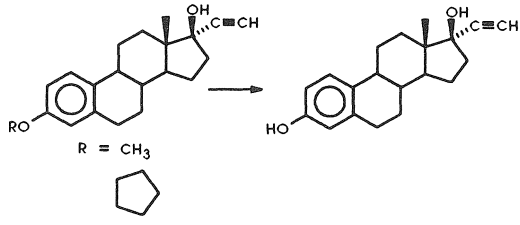


Figure 8. Metabolites of 3-0-alkyl ethers of estrogens proceeds largely via 0-dealkylation followed by normal phase I and phase II transformations.

Therapeutically this compound has the advantage of a greater degree of stability when taken orally. This factor is less significant when an intravenous route is employed, since the first transformation that occurs is the cleavage of the methyl ether to yield the free phenolic estrogen. The subsequent steps proceed the same as observed with the parent compound. The methyl ether moiety primarily alters the initial distribution of the drug such that relatively less goes initially to the target tissue and more to the fat. The affinity of the ether for the receptor is much less than the parent compound, however, as the ether moiety is cleaved, a gradual accumulation in the target tissue is observed (51). Thus the compound functions as a pro-drug. The rate of this cleavage can be retarded by employing a larger or more bulky alkyl group such as the cyclopentyl group found in quinestrol (50). In this case, urinary excretion is low, on the order of 15-20% over six days in humans.

4. 17α-ETHYNYL-11β-METHOXYESTRADIOL [MOXESTROL] 4

Moxestrol is one of the most potent estrogens in animals and humans (51). It owes its effectiveness both to its relatively high affinity for the estrogen receptor and the relatively low associations with the sex steroid binding (SSBG) globulin in the plasma. The drug is, therefore, more available to the target cells and its slow dissociation rate from the receptors effectively traps it there. These factors are assisted by the substitution at the 17α-position that retards the rate of metabolic deactivation compared with estradiol. The analysis of the metabolites in several species, including man, provides the profile shown in Figure 9 (64,65). The metabolism that does occur has now been redirected toward the D-ring where one observes 16α- and 16β-hydroxylation as well as 15α-hydroxylation. The hydroxylation which was extensive for ethynyl estradiol is reduced to a few percent of the urinary metabolites, which are the glucuronide and sulfate conjugates. Apparently, the

11β-methoxy group, in addition to suppressing SSBG binding, also retards the aryl hydroxylation reaction. An unusual metabolite consisting of an expanded D-ring has been identified and probably arises from the oxidation of the ethynyl moiety (65).

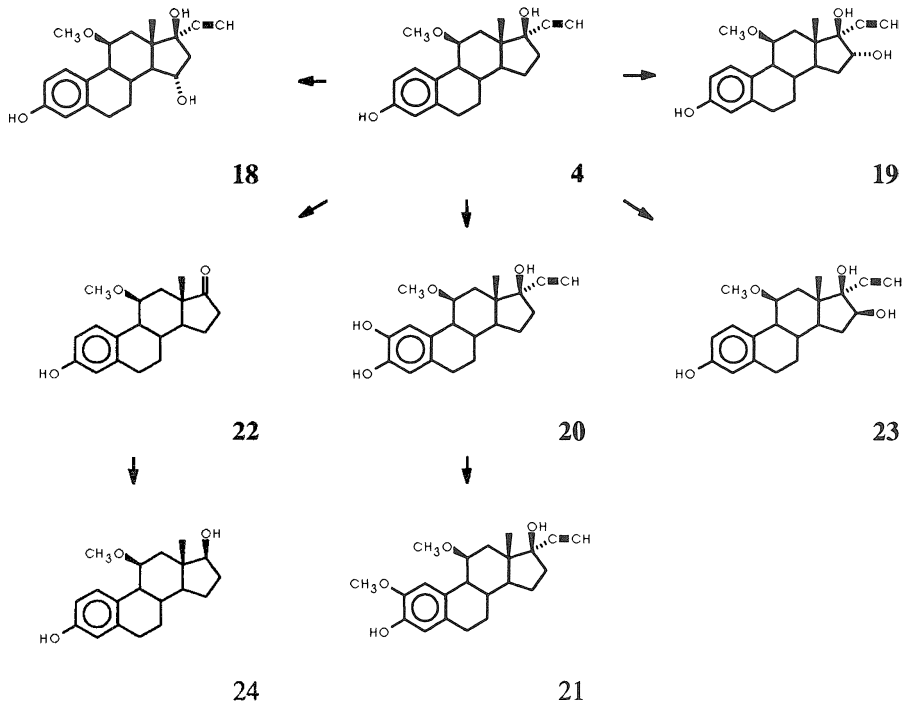


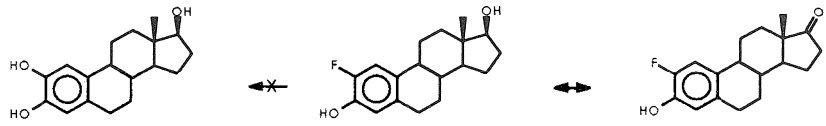
Figure 9. Summary of major phase I transformations of moxestrol 4.

C. Potential Metabolic Pathways for the Radiohalogenated Estrogens

1. A-RING SUBSTITUTED RADIOLABELED ESTROGENS

The A-ring substituted estradiol derivatives were among the first to be prepared, primarily because the introduction of a radiohalogen could be readily accomplished via electrophilic substitution ortho to the phenolic -OH. The synthetic problems that arose, however, resulted from a lack of regiospecificity, i.e., both 2- and 4-halo products were prepared, as well as

dihalogenation. It was soon apparent that the introduction of a large halogen such as bromo or iodo group at either the 2- or 4- position caused a marked decrease in receptor affinity (16). For the iodinated agents, there was also a dramatic increase in nonspecific binding, in particular to thyroxine binding globulin (TBG). Therefore, the only A-ring agents currently of interest are the 2- and 4-fluoroestradiol (37,38), which retain receptor affinity and the 1-halo-estradiols reported a few years ago by Hylarides et al (44,45), which also retain good affinity for the estrogen receptor. The potential effect of the substituents upon the metabolic transformation of the estrogen are shown in figures 10 through 12.



12 25 26

Figure 10. Presence of 2-fluorosubstituent suppresses formation of catechol estrogens, e.g. 12.

The effect of 2-fluoro substituent will be most apparent on A-ring metabolism. Oxidation at the 2-position normally yields the 2-hydroxy estradiols (catechol estrogen), however, the presence of a strong electron withdrawing group effectively suppresses this reaction (66). As a result, both the levels of catechol estradiol and its 0-methyl conjugate (2-methoxy estradiol), would be substantially reduced. A second reaction at the A-ring, that of sulfate conjugation by the sulfotransferase enzyme (67), would not be inhibited and in fact, may be enhanced due to the acid strengthening effect of the 2-fluoro group.

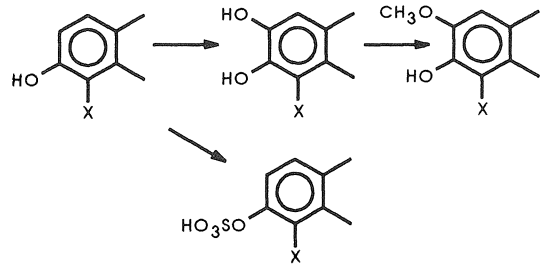


Figure 11. Presence of 4-halo substituent modifies A-ring phase I and phase II reactions.

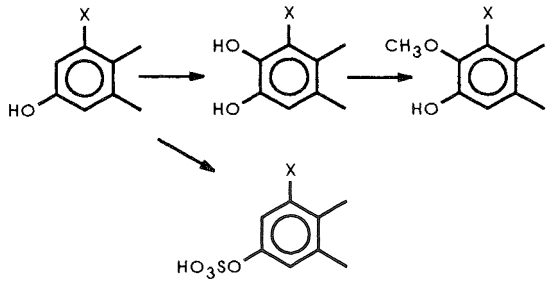


Figure 12. Presence of 1-halo substituent modifies transformations at 2- but not 3- position of estrogens.

In general, the presence of a 4-fluoro or a 1-halo substituent on the estradiol nucleus would not be expected to cause as significant an alteration in the metabolic profile as the 2-fluoro moiety. This would arise from the observations that the primary A-ring metabolic reactions occur at the 2- and 3-positions. The oxidation to the catechol estrogen may proceed through direct hydroxylation rather than via an arene oxide and therefore, the substituent at the 1-position would play a relatively minor role in altering that reaction (68). Also catechol-0-methylation at the 2-position would not be significantly affected. A 4-fluoro group would modify neither of the above reactions significantly nor would it be expected to inhibit the sulfotransferase conjugating enzyme for the same reasons.

2. D-RING SUBSTITUTED RADIOLABELED ESTROGENS

The majority of the newer radiolabeled estradiol derivatives have incorporated the radionuclide on the D-ring of the steroid nucleus. This has been the result of structure-activity relationship studies that have demonstrated that the introduction of certain substituents at the 16α- and or 17α-position can be accomplished without seriously diminishing the estrogenic activity of the product (11,13,14,35-37,69-72). Radiochemists have developed newer methods for rapidly introducing the short-lived radionuclides, such as C-11, F-18 and I-123, and purifying the reaction mixtures in a time frame consistent with clinical uses (28-31, 39, 40,41). The products that have been developed, however, have not been explicitly examined with respect to their potential metabolic transformations. The following sections will evaluate the two classes of radiolabeled estrogens, the 16α-halo-estrogens and the 17α-substituted derivatives, with respect to the potential effects of those substituents upon metabolism.

The 16α-halogenated estradiol derivatives will most closely resemble the corresponding estriols in their metabolic transformation. The position of the 16α-halogen (F, Br or I), is the same as the orientation of the hydroxyl in estriol (74) and therefore, would not be expected to cause a major divergence in most metabolic reactions as shown in Figure 13. For all practical purposes A-ring reactions such as aromatic hydroxylation and conjugation reactions will be unaffected. The perturbation of D-ring oxidations are somewhat more speculative.

The oxidation of the 17β-hydroxyl of these labeled steroids yields the corresponding 16α-haloestrones. There may be some modification of the rate at which this reaction occurs but it should proceed in the same order of magnitude as estradiol and estriol. The chemical stability of the product is reduced because one now has an α-haloketone and the reactivity of such

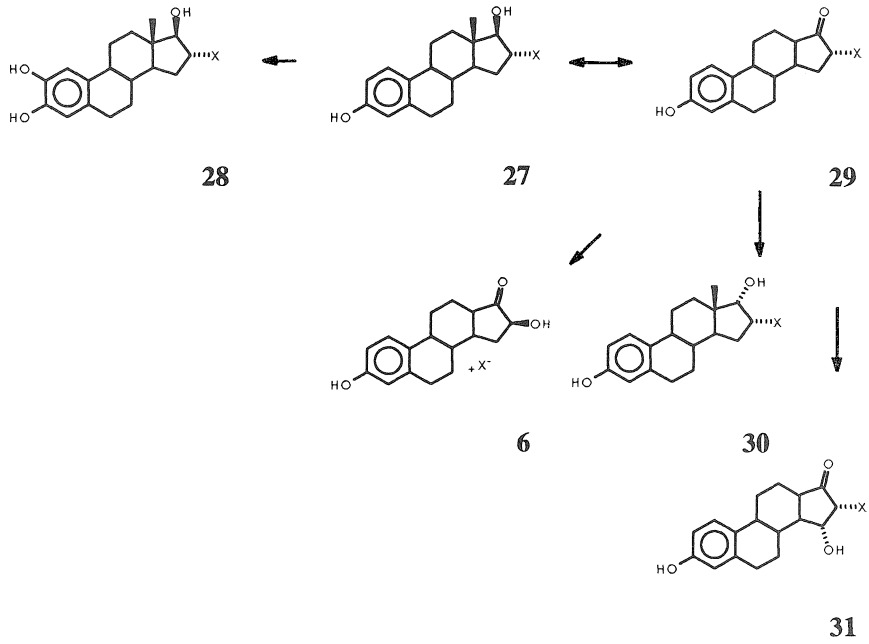


Figure 13. Examples of phase I metabolic transformations for 16α-haloestradiol 27.

compounds toward nucleophiles is well known. The 17-ketone can be reduced to yield the 17α-epimer or can be hydroxylated at the 15α-position. Subsequent metabolism provides the polyhydroxy estrogens which are substrates for the Phase II glucuronate and sulfate conjugation enzymes. Aqueous hydrolysis is one transformation that occurs with the alkyl halides that does not require metabolic action. This would be more prevalent with the

16α-iodo and bromo derivatives than with the 16α-fluoroestradiol because of the weaker C-I and C-Br bonds. Loss of the radiohalide would be more noticable with the iodine because free iodide is readily accumulated by the thyroid, whereas free bromide is readily excreted in the urine. Although the fluoride ion is sesquestered in bone, one would expect the amount of free ion to be low.

Therefore, one would not expect that the introduction of a 16-halo substituent to provide enhanced stability for the labeled estrogen toward metabolic processes. However, the addition of an 11β-methoxy group, as seen with 16 [Br-77]-bromo-11β-methoxyestradiol, in addition to affecting the plasma protein binding component in the biological distribution also has some effect on the rate of D-ring metabolism.

The 17α-labeled substituents that have been prepared or proposed have included methyl [C-11] (41), ethynyl [C-11] (40), haloethynyl [I-123, Br-77] (16,17,32), and halovinyl [I-123, Br-77, F-18] (28-31, 33,39,75). The first two, i.e., 17α-methyl and 17α-ethynyl estradiol, would undergo metabolic transformations identical to those of the corresponding unlabeled parent compounds. One would expect analysis of the metabolites from these derivatives to show a preponderance of conjugates of the parent compound accompanied by metabolites arising from 2-, 17- and 16-hydroxylation, the latter two being minor for the 17-ethynyl estradiol (Figure 14), i.e., the Phase I transformations are directed primarily toward A-ring oxidation at the 2-position. Oxidation at the 15- and 16-positions will dominate the metabolite profile. Some of the 2-methoxy derivatives arising from the catechol estrogen will also be present.

The 17α-substituent, as has been noted before, can also undergo transformations, both chemical as well as biochemical. The C-halogen bond in the ethynyl-iodide and bromides is not strong and can undergo dissociation. This may account for the instability of the 17α-bromoethynyl and 17α-

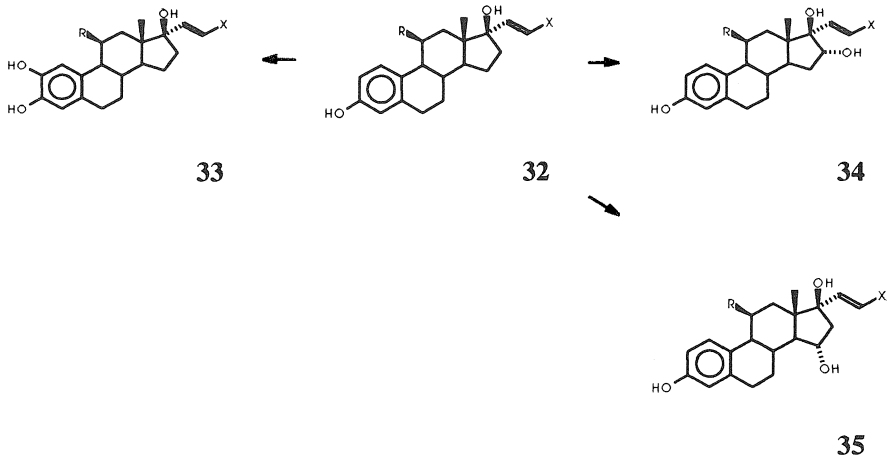


Figure 14. Potential phase I metabolic pathways for 17α-halovinyl-estradiols 32.

iodoethynyl estradiols prepared and examined by Eckelman et al (17,32). The 17α-halovinyl estrogens may undergo oxidative reactions similar to those reported by Ortiz de Montellano et al for vinyl chlorides (76-78). Such groups yield reactive intermediates that can irreversibly inhibit the enzyme. The extent to which such reactions would occur with the 17α-halovinyl estrogens depends upon the competition between the Phase I and Phase II processes at the D-ring.

D. Metabolic Studies on Radiolabeled Estrogen

It is unfortunate that there have been so few studies performed with the single photon- or positron-emitting estradiol derivatives with the objective of

examining the metabolic pathways. A review of the literature in this area does provide some instances in which the presence of radioactive parent drug, as a percentage of total radioactivity is present in the tissue and/or plasma has been determined. For example, both Symes, et al (79) and Hochberg, et al (80) evaluated radioiodinated 16α-iodoestradiol as a radiotracer for the estrogen receptor in the hormone responsive breast tumors. In the former study using I-131, imaging results were poor, although the latter study with I-125 confirmed the nuclear uptake of the agent. Both groups observed rapid metabolism of the radiopharmaceutical leading to low tissue to blood ratios. During the period from 5 to 90 after administration the 16α-[I-125,131] iodoestradiol in the blood compared to total plasma radioactivity declined from 90% at 5 min. to 5-10% at 90 min. The radioactivity also underwent extensive enterohepatic recycling, consistent with the metabolism observed in other species, and a half-life of approximately 36h was determined. Although an extensive analysis of the metabolites was not undertaken, a less polar material appeared in the plasma which remained relatively constant whereas the level of parent agent declined. The authors speculated that this may be the 16α-[I-131] iodoestrone, but this was based solely on chromatographic properties and not substantiated by any other methods.

Similar studies with 16α-[F-18] fluoroestradiol have been conducted in rats (39-81). Again, a rapid conversion of the parent compound to its metabolites was observed such that the preponderence of the circulating activity consisted of metabolites. An analysis of the uterus indicated that virtually all of the activity that was present was the parent estrogen. If one compares the ratio of parent estrogen in the target to that in the plasma, very high ratios were obtained. However, the significant levels of radioactivity in the plasma resulting from metabolism reduce the actual radioactivity ratios. Another potential problem with this labeled estrogen relates to the gradual release from the target tissue via dissociation from the receptors. As an

estradiol/estriol analog it has a significant dissociation rate vis a vis the 17α-alkynyl estrogens and therefore returns to the plasma compartment to be subject to metabolism. Without a 17α–substituent to retard both receptor dissociation and metabolism the clinical utility of this agent may be comprised by the constellation of labeled metabolites that are produced.

Hanson, et al have conducted preliminary studies with the 17α-[I-125]iodovinyl-11β-methoxyestradiol 3-0-methyl ether (IVβ–Me_2-3OMe), a radiopharmaceutical that demonstrated promising results in immature rats (31). In this pilot project, the radiochemical was injected intravenously and rats were sacrificed at 0.25, 1.0 or 60 h post injection. The plasma and uteri were isolated and analyzed for the presence of parent compound IVβ–Me_2-3OMe and the putative metabolite IVβMe_2. First, both the plasma and tissue were extracted to determine the percent of total radioactivity that was organic soluble. As Figure 15 indicates, at each of the time points evaluated 95-98% of all the activity in the tissue was extracted.

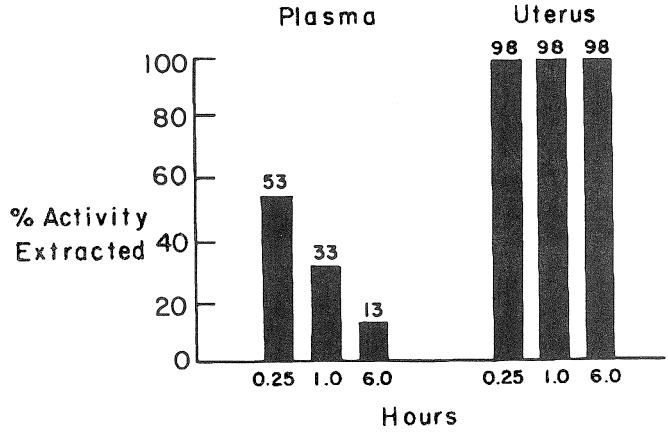


Figure 15. Organic solvent soluble radioactivity in plasma and uterus following i.v. administration of 17α[I-125]iodovinyl-11β-methoxyestradiol-3-0-methyl ether.

For the plasma, however, the percent of radioactivity associated with neutral organic compounds declined from 50-55% at 0.25h to 30-35% at 1h to 10-15% at 6h. This is similar to that observed with 16α-[I-125] iodoestradiol except the decline for IVβ-Me_2-3OMe is slower and the amount radioactivity in the plasma is quantitatively much lower. The identity of the compounds in the organic extracted fraction was evaluated by cospotting unlabeled IVβ–ME_2-3OMe and IVβME_2 on the TLC plates. Virtually all of the radioactivity comigrated with one or the other of these compounds. As Figure 16 demonstrates, IVβME_2 begins to appear in both the plasma and tissue by 0.25h post injection, accounting for 35-50% of the total activity, the balance being primarily the parent radiochemical.

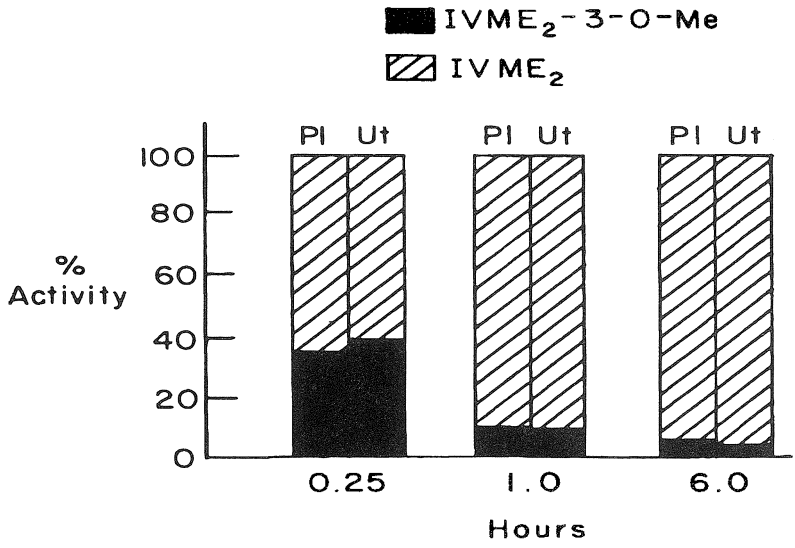


Figure 16. Distribution of radioactivity in organic soluble fraction between parent compound and its 0-demethylated metabolite.

By 1h, however, the parent radiochemical comprises only 10% of the total radioactivity and 90% is the 0-demethylated metabolite. This proceeds further

such that by 6h over 95% of the radioactivity in the organic soluble fraction consists of the IVβME$_2$ and only about 5% is the parent compound. The results, while not definitive, suggest that the estrogen nucleus, when substituted with 11β- and 17α-substituents is relatively stable toward most of the oxidative metabolic tissue formations. The bulk of the metabolites may result from the Phase II conjugative reactions which would be observed with any or all of the radiolabeled estradiol derivatives. An interesting aspect of the above study was the effect of the 3-0-methyl ether. Its presence negates the sulfation of the 3-hydroxyl which is a major metabolic pathway for the estrogens. Hepatic 0-dealkylation yields the free phenolic estrogen which can either diffuse into the target tissue (along with nontarget tissue) or undergo conjugation. The gradual accumulation of radioactivity observed in the initial biodistribution work and the subsequent metabolism study suggests that the methyl ether converts the compound into a prodrug that slowly releases the more potent estrogen which can successfully bind to the target receptor.

II. SUMMARY

The area of radiopharmaceutical chemistry has progressed remarkably in the development of single-photon or positron emitting estradiol derivatives for imaging hormone responsive breast cancer. As the best agents, whether labeled with I-123, C-11, or F-18, undergo clinical trials, an increased emphasis is going to be placed upon the pharmacokinetics of the parent agent and the appearance and disposition of metabolites. A review of the studies performed with [H-3] and/or [C-14] labeled estrogens and subsequently with radiohalogenated or [C-11] labeled steroids indicates that certain substitution patterns are more likely to produce successful results than others. In particular, the presence of a 17α-substituent is desirable because of its ability to suppress the Phase I transformations that occur on the D-ring, especially the oxidation of the 17β-hydroxyl and the 16α-and 15α-hydroxylations. The introduction of

an 11β-group has two major effects, first to reduce protein binding in the plasma but also secondly to retard the rate of metabolism in the D-ring. The presence of a small group at the 2- or 4-position of the A-ring tends to inhibit the formation of the catechol estrogens and in this regard the 2-position is favored over the 4-position. Lastly, it may be to some advantage to cap the phenolic OH with a readily metabolized group to provide the more active agent gradually over time. This effect, however, is more speculative than the others. As a result, one can depict an "ideal" estrogen as shown below in which most of the modifications suggested have been incorporated. The task is now to prepare and evaluate it (Figure 17).

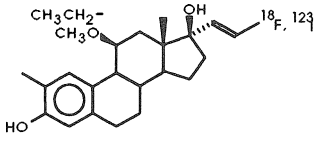


Figure 17. The structure of the "ideal" labeled estrogen 36 contains the 2-fluoro group to suppress cathechol formation, the 11b-substituent to retard A- and D-ring metabolism and to reduce plasma binding, and a 17a-halovinyl group to stabilize the structure toward D-ring metabolism and in vivo dehalogenation.

Acknowledgements

This work has been supported in part by grants from NIH, CA31624 and CA41399, and a contract from DOE, DE-FGO2-86, ER60560.

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Renal Radiopharmaceuticals

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I. INTRODUCTION

During the past thirty years numerous radiolabeled compounds have been proposed and evaluated as potential tracer agents for the radionuclide study of renal function and morphology. Starting with radioiodinated contrast agents in 1952 [1] and radiomercury compounds in the early sixties [2] - both groups characterized by a significant radiation burden to the patient - one has arrived now at a group of ^{99m}Tc labeled renal tracer agents with a low radiation dose and specially designed for the study of different specific renal parameters. The extensive search for suitable renal radiopharmaceuticals can be understood from the viewpoint that the kidney is a vital organ responsible for the maintenance of composition of blood and extracellular fluid and thus deserves optimal diagnostic agents for its evaluation. The high number of tested substances can also be ascribed to the fact that almost any polar compound with a molecular weight up to 500 is handled at least partly by the kidneys and thus constitutes a potential agent to furnish information about function and/or morphology of these organs.

Radionuclide imaging is probably not a first-choice technique for the accurate determination of size, site and shape of the kidney or visualization of morphological abnormalities [3,4], but is ideally suited for the noninvasive evaluation of renal function. By the proper choice of the most appropriate radiopharmaceutical and the use of suitable equipment radioisotopic renal studies allow a rapid determination of functional parameters such as glomerular filtration rate and effective renal plasma flow, together with quantitation of functional renal mass, postvoiding residual urine volume and vesico-ureteral reflux. Additional information about renal function that can be obtained easily by these techniques includes the measurement of differential and total renal function, the mean parenchymal and whole kidney transit time, the minimum transit time, the parenchymal transit time index and evidence of significant outflow resistance as shown by the response to furosemide [5].

These studies can be done with minimal discomfort to the patient and an acceptable or often negligible radiation dose.

This chapter provides an overview of the most important tracer agents for renal studies which have been used in the past or are currently in use. Special attention will be directed to the relation between chemical structure and physical characteristics of the radioactive drugs and their biodistribution and renal handling. In this way it is both an updating of previous review papers on this subject [3,4,6-12] and an attempt to further disclose the underlying biological mechanisms and structural requirements for renal radiopharmaceuticals with quite diverging biological fate.

II. RENAL ANATOMY AND PHYSIOLOGY AS RELATED TO THE BEHAVIOR OF RENAL RADIOPHARMACEUTICALS

Our terrestrial mode of life is greatly dependent on the constancy of the aqueous internal environment in which the cells of the body live and carry out their activities. This constancy is achieved by the excretory and regulatory function of the kidneys as they regulate not only the volume but also the composition of the extracellular fluid compartment [13]. To perform this dual function the kidneys each contain approximately 1.3 million structural and functional units, termed nephrons. The principal segments of the nephron involved in the handling of renal radiopharmaceuticals are the glomerular capillaries and the proximal tubular cells. Figure 1 shows the morphology of the nephron and its localization with respect to the gross renal anatomy.

The kidneys of a resting adult receive each minute approximately 1.25 l of arterial blood, just under 25% of the cardiac output, via the afferent arterioles of the juxtamedullary and cortical nephrons. Of this amount 16% to 20% is filtered by the glomeruli, a tuft of capillaries invaginated into Bowman's capsule, the dilated blind end of the nephron. The permeability of these

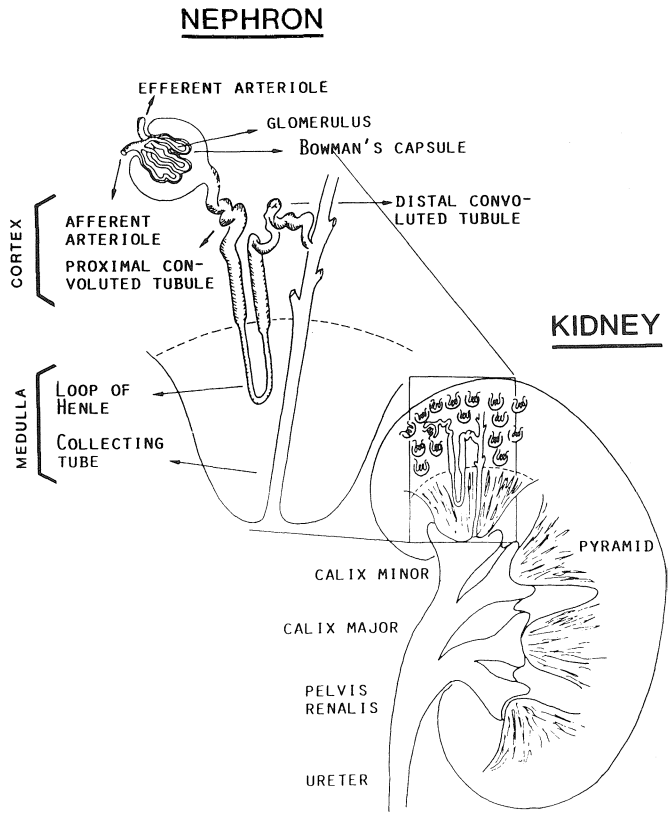


Figure 1. Structural Segments of the Nephron and General Anatomy of the Kidney.

capillaries is 50 to 100 times that of the capillaries in skeletal muscle. The resulting ultrafiltrate of blood has a similar composition to plasma except for the extremely low concentration of lipids and proteins with a molecular weight over 70,000 daltons for which glomerular filtration is minimal.

As this filtrate passes along the proximal convoluted tubule about 75% of the solutes are reabsorbed into the plasma by diffusion, endocytosis or carrier-mediated transport. A similar quantity of the filtered water is returned to the general circulation and extracellular fluid by osmotic pressure. On the other

hand some substances are added to the filtrate by active or passive tubular secretion from the peritubular capillaries to the tubular fluid. Reabsorption by simple diffusion down chemical gradients is possible for all kind of compounds but is most significant for highly lipid soluble substances. The lipid solubility of weak acids and bases depends on their degree of ionization and this explains the importance of urinary pH for their excretion [14]. Small proteins and some peptide hormones are reabsorbed by endocytosis [15]. A number of other constituents of the glomerular filtrate including glucose, hydroxy acids, amino acids, and sodium and bicarbonate ions are reabsorbed by carrier-mediated transport. Active transport of compounds from an area of lesser to an area of greater concentration requires energy expenditure. It is also characterized by a maximal capacity of the transporting mechanism and susceptibility to interference by competitive inhibitors. It was detected many years ago that the L-forms of amino acids are reabsorbed to a much larger extent than are the D-forms [16].

Apart from reabsorption the renal tubules are capable of transporting materials from the peritubular fluid to the tubular lumen by similar mechanisms of passive diffusion and active secretion. Distinct secretory mechanisms which exhibit an absolute limit of transport capacity have been described : the system responsible for transport of organic acids such as hippurates, penicillins and glucuronides and a second system for strong organic bases including guanidine, choline and histamine [17]. Little is known about a possible third system characterized by its ability to secrete EDTA, but its role in human renal function seems to be of little importance [18].

The soluble cytoplasmic fraction of the proximal tubular cells contains metallothionein, a low molecular weight protein (relative molecular weight about 10,000) with a high sulfhydryl content, principally as cysteine (about 26 SH groups per mole of protein) [19,20]. Heavy and transition metals such as cadmium and mercury reabsorbed after filtration through the glomeruli or

diffused directly from the peritubular capillaries are bound firmly to this protein and may be stored for a prolonged period in the proximal tubular cells. This mechanism of renal fixation is probably involved in the renal accumulation of radiomercury [12] and some ^{99m}Tc radiopharmaceuticals.

During the passage of the tubular fluid through Henle's loop and the distal convoluted tubule another 10% of the filtered water is removed and electrolytes are actively reabsorbed. This process is continued in the collecting ducts. Finally 99.7% of the filtered water is reabsorbed and the rest is excreted together with waste products and excess materials in the form of urine. It is not clearly demonstrated whether tubular reabsorption and/or fixation of some radiopharmaceuticals also happens at the distal tubular cells as proposed by some authors [3,6]. Henle's loop and the collecting duct do not seem to play an important role in the excretion or fixation mechanism of these agents. The different pathways that can be followed by renal radiopharmaceuticals can be summarized as follows :

i) compounds strongly bound to plasma proteins with a molecular weight over about 60,000 behave as the protein and are neither filtered through the glomerular capillaries [4], nor diffuse out of the peritubular capillary network. Protein molecules with a molecular weight of 30,000 or less appear in the glomerular filtrate and are reabsorbed intact by the proximal tubules in the cortex by endocytosis [21]. It can be assumed that ^{99m}Tc-aprotinin behaves in this way.

ii) the non-bound fraction of polar low molecular weight compounds (e.g. complexes of ^{99m}Tc with DMSA, DTPA, gluconate) is filtered through glomerular capillaries into the tubular lumen or diffuses from the peritubular capillaries into extracellular fluid surrounding tubular cells. Establishment of a new equilibrium between bound and non-bound fraction in the plasma will result. In humans, the total area of glomerular capillary endothelium across which filtration occurs is about 0.8 m^2. The total surface of the renal

capillaries is approximately 12 m^2 [15]. For this reason more molecules can leave the plasma at the tubular capillaries than at the glomeruli.

iii) molecules in the glomerular ultrafiltrate can be transported to the bladder with the urine (e.g. ^{99m}Tc-DTPA) or diffuse into renal tubular cells (e.g. ^{99m}Tc-DMSA, TcO_4^-).

Three different pathways are then possible

- diffusion across the cell and passage from the extracellular fluid into an adjacent capillary (reabsorption by diffusion).
- interaction with a macromolecule in the renal cell followed by temporary or prolonged retention in the cell (tubular fixation),
- uptake by a carrier, transport across the cell and release into the extracellular fluid (reabsorption by carrier-mediated transport)

iv) molecules that have diffused from the peritubular capillaries into the extra cellular fluid can be picked up at the membrane of the renal tubular cells by a carrier or enter these cells by diffusion. Then they are transported across the cell into the tubular fluid and excreted with the urine (carrier-mediated tubular secretion, e.g. radioiodinated hippurate, ^{99m}Tc-MAG_3) or they remain bound to the carrier or other macromolecule within the cell (renal tubular fixation, one of the pathways proposed for ^{99m}Tc-DMSA)

Table 1 lists the average values of some important renal function parameters that can be assessed by radioisotopic studies.

III. RENAL RADIOPHARMACEUTICALS

A limited number of radiolabeled substances follow a single renal pathway, namely glomerular filtration without tubular reabsorption, and these compounds are useful for estimation of the glomerular filtration rate (GFR). Other tracer agents with a more complex renal handling can nevertheless also be used in radionuclide renal studies if their renal behavior is sufficiently established to correctly interprete the imaging data. This holds true for

Table 1. Mean Values in Adult Humans for Renal Functional Parameters Assessed by Radionuclide Studies.

PARAMETER	MEAN VALUE (Men)	CALCULATION
Glomerular filtration rate (GFR, clearance of inulin)	120 ml/min	$U_{In}V/P_{In}$ or $D/\int_0^\infty P_t \cdot dt$
Effective renal plasma flow (ERPF; clearance of PAH)	630 ml/min	$U_{PAH}V/P_{PAH}$ or $D/\int_0^\infty P_t \cdot dt$
Actual renal plasma flow (RPF)	700 ml/min	ERPF/extraction ratio PAH
Renal blood flow (RBF)	1250 ml/min	RPF/1-Hematocrit
Urine Production rate	0.9 ml/min	(measured)

U: concentration of inulin (In) or para-aminohippuric acid (PAH) in urine.

V: urine flow per unit of time.

P: mean concentration of inulin or PAH in plasma.

D: injected dose.

P_t: concentration of tracer agent in plasma at time t.

radiopharmaceuticals used in the quantitation of functional renal mass or in the evaluation of the tubular secretory function.

A. Radiopharmaceuticals with Significant Retention in Kidneys

Radionuclide renal imaging to gain anatomical information is not a first-line procedure but should be performed to complement information readily available from alternative morphologic imaging methods. However static renal imaging with a tracer agent that is partially retained in the kidneys can also provide an estimation of relative renal mass and indicate whether an intrarenal mass is a tumor or functioning renal tissue. Other clinical conditions in which such studies can be helpful are summarized by Blaufox et al. [22].

A radiopharmaceutical is most suitable for this kind of study the more it is retained in the functioning renal tissue with minimal uptake in other organs. The usefulness of such tracer agent is however also greatly determined by other parameters such as radiation dose to the patient, imaging characteristics of the radionuclide, time of maximal fixation, ease of preparation, stability and reproducibility of the study. The mechanism of retention of these metals in the kidneys is not clearly understood but is thought to be via ligand exchange and subsequent strong binding of the metal to immovable ligands in the kidney tissue.

1. RADIOMERCURY COMPOUNDS

Intravenously administered inorganic and organic mercury compounds bind significantly to the metal binding protein metallothionein within the proximal tubular cells [23-26]. On this basis a number of radiomercury compounds has been used extensively for determination of functional renal mass [27-30]. However, they are now completely abandoned in favour of ^{99m}Tc compounds which are characterized by more favorable imaging characteristics and a much lower radiation dose.

2. ^{99m}Tc-LABELED RADIOPHARMACEUTICALS

Technetium-99m is the prefered radionuclide in nuclear medicine due to its attractive physical properties, nearly ideal for clinical imaging, and its continuous availability in almost unlimited amounts in any nuclear medicine department. Many ^{99m}Tc-complexes have been reported to show significant uptake and retention in the kidneys and were proposed as potential radiopharmaceuticals for morphological kidney imaging or determination of functional renal mass. These agents include complexes of ^{99m}Tc with poly-alcohols, hydroxy acids, mercapto acids, polypeptides, antibiotics and phosphonates (Table 2).

Table 2. ^{99m}Tc-Complexes Studied as Potential Morphological Kidney Tracer Agents.

Complex of ^{99m}Tc with	References	Complex of ^{99m}Tc with	References
- sorbitol, mannitol	31,32	-dimercaptopropionic acid	61
- inulin, N-methylglucamin	31,32	- 2,3-dimercaptopropane sulphonic acid	63,64
- iron-ascorbic acid	32	- dimercaptosuccinic acid	57
- ferric ascorbate-DTPA	33	- dimercaptoglutaric acid	62
- gluconic acid	34,35	- dimercaptoadipic acid	62
- lactobionic acid	32	- gelatin	106
- glucoheptonic acid	32	- caseidin	107
- malic acid	36	- glutathione	108
- citric acid	37	- tetracycline	114
- penicillamine, cysteine	41,50-54	- gentamycin	1165116
- penicillamine-acetozolamide	55,56	- phosphomycin	117
- acetylcysteine	41,58	- ethylthiomethyl phos-phonate	118
- thiomalic acid	41,59,60	- pyrophosphate	119
- thiolactic acid	41	- methylene diphosphonate (MDP)	120,121

None of these complexes is taken up in the kidneys entirely and considerable variation is noted in the rate and degree of uptake and duration of

retention between the various agents. Many of these ^{99m}Tc complexes have been tested in humans and a limited number are now in clinical use.

a) COMPLEXES OF ^{99m}Tc WITH POLY-ALCOHOLS AND HYDROXY ACIDS

Such complexes are relatively weak and for this reason susceptible to exchange in the presence of e.g. sulfhydryl compounds. On this basis ^{99m}Tc-hydroxy acid complexes such as ^{99m}Tc-gluconate, -malate, -lactate, -tartrate, α-hydroxybutyrate and -citrate have been used or proposed as intermediate complex in the exchange labeling of newer ^{99m}Tc-radiopharmaceuticals [e.g. 38-42]. A similar ligand exchange can be supposed to take place with strongly chelating proteins such as metallothionein in the proximal tubular cells. After diffusion or transport of the ^{99m}Tc-complexes into the tubular cells from the nephron lumen following glomerular filtration or from the capillaries perfusing the renal tubules, competition between metal binding macromolecules within the tubular cells and the poly-alcohol or hydroxy acid would result in a transfer of cationic ^{99m}Tc to the macromolecules. No evidence has been found, however, to show whether these ^{99m}Tc complexes are dissociated or withheld as such in the renal cortical cells.

A preparation containing ferric ascorbate-DTPA was the radiopharmaceutical of choice in the U.S. for many years [10] and is still described in U.S.P. XXI. Complexes of ^{99m}Tc with gluconic or glucoheptonic acid are now commonly used. Intravenously administered ^{99m}Tc-gluconate is rapidly cleared from the blood by glomerular filtration and then partially reabsorbed by the renal tubules [43]. The activity localized in the renal cortex is maintained at a significant level (about 12% from 1 hr - 24 hr p.i.) by re-equilibration of the fraction in the extracellular fluid [35].

From a strictly technical standpoint the data obtained with ^{99m}Tc-gluconate are similar to those given by the radiomercury compounds [44], but the absolute renal uptake is lower and the renal pathway is different.

The chemistry and biological behavior of ^{99m}Tc-glucoheptonate have been studied in detail. Stannous ions are used as the reducing agent in the commercial labeling kits but preparations with similar physical and biological properties are obtained using electrolysis [45], formamidine sulfinic acid [46] or sodiumborohydride [47] for the reduction, which indicates the absence of Sn in the complex. The complex contains a Tc = 0 core and two glucoheptonate ligands, bidentate bound to Tc by the oxygens of the end carboxyl group and the adjacent hydroxyl group [47].

In a study in rats, Lee and Blaufox [48] observed that kidney uptake of ^{99m}Tc-glucoheptonate is significantly reduced by probenecid blockade and para-aminohippuric acid (PAH) competition. They suggest that this radiopharmaceutical is cleared by two mechanisms: (a) tubular secretion of the protein bound fraction (46% of injected dose at 5 min p.i.) by the same carrier system involved in PAH and hippuric acid transport and (b) glomerular filtration of protein free portions. Renal accumulation in humans was found to be maximal at 2 hr after injection and ranged from 19.1 to 25.5% of injected dose in 10 patients [49].

b). COMPLEXES OF ^{99m}Tc WITH MERCAPTO ACIDS

A sulfhydryl group is far more nucleophilic than an aliphatic hydroxyl group and therefore the metal-sulfur bond is much stronger than the metal-oxygen bond. Complexes of ^{99m}Tc with mercapto carboxylic acids are, as a consequence, relatively stable and more difficult to dissociate than the hydroxy carboxylic acid complexes.

The mechanism of renal accumulation of thiolate-containing tracer agents is not clearly understood. Their affinity for proteins is evident, as shown by

their high protein binding in plasma from 82-97% [41], but knowledge about the exact renal pathway is lacking.

In 1974 Lin et al. [57] introduced the complex of ^{99m}Tc with dimercaptosuccinic acid (DMSA), characterized by a renal uptake comparable to that of the radiomercurials and a remarkably low urinary excretion, respectively 54% and 7% of the injected dose at 1 hr p.i. in rats.

^{99m}Tc-DMSA is currently a routine first-choice radiopharmaceutical for quantitative or visual determination of differential renal function [65-71]. The mean renal uptake of ^{99m}Tc-DMSA at 3 hr p.i. in normal humans is about 50% of the administered dose [72-74] while 10% to 20% is excreted in the urine during the first 2 hr after injection [3,74]. Plasma clearance in 13 normal volunteers with normal renal function was 34 ml/min and urinary clearance 12 ml/min [86]. ^{99m}Tc-DMSA localizes specifically in the proximal convoluted tubule [69] where it binds mostly to soluble cytoplasmic proteins and mitochondria, and to a lesser extent to both microsomes and nuclei [75-77].

The preparation and handling of ^{99m}Tc-DMSA require special attention as the composition, stability and renal uptake are affected by a number of parameters. Depending on pH, relative amount of reactants and incubation time four distinct ^{99m}Tc-DMSA complexes with a different biodistribution are formed [78-81]. Labeling at pH 2.5 is required to obtain the complex with reproducibly high renal cortical affinity, optimal for renal imaging. There has always been a lot of speculation about the exact structure of this ^{99m}Tc-DMSA complex and hard evidence such as X-ray crystallographic data has never been published. One assumes that it is a hexacoordinated $Tc(DMSA)_2$complex with Tc in the trivalent state [80-82]. Moretti et al. [80,83,84] suggest that Tc is bound to two sulfur atoms and one carboxyl-oxygen of the first DMSA molecule and to two carboxyl-oxygens and one sulfur atom of the second ligand molecule (Figure 2).

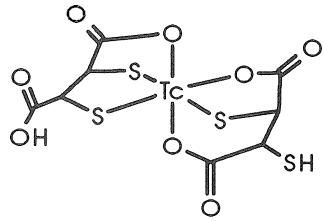


Figure 2. Structure of $Tc(DMSA)_2$ as Proposed by Moretti et. al. [80,83,84].

Because of the high plasma protein binding of ^{99m}Tc-DMSA (values varying from about 73% to 97% in humans have been reported [85,86]), it has been suggested that ^{99m}Tc-DMSA might reach the proximal tubular cells mainly directly from the peritubular capillaries [3,87,88]. There is however growing evidence against this hypothesis

i) kidney uptake or urinary excretion of ^{99m}Tc-DMSA is not affected by probenecid blockade [48,87,88], indicating that the hippurate transport system is not involved in ^{99m}Tc-DMSA transfer,

ii) blockade of glomerular filtration by captopril treatment in a kidney with arterial stenosis resulted in disappearance of ^{99m}Tc-DMSA uptake, while hippurate uptake and accumulation took place [89,90],

iii) urinary excretion of the tracer is increased in cases of proximal tubular dysfunction [90,92].

De Lange et al. [86,93] conclude from their results that peritubular uptake accounts for about 65% and glomerular filtration followed by endtubular reabsorption for about 35% of the renal handling of ^{99m}Tc- DMSA. Peters et al., however, suggest that the majority of the ^{99m}Tc-DMSA activity that becomes fixed in the renal cortex is reabsorbed from tubular fluid after filtration at the glomerulus [94].

Labeling of DMSA with technetium-99m at pH 8 yields a high molecular size polynuclear Tc(V)-DMSA complex that is supposed to contain a TcO_4^{3-}

core [95,96]. Its biological characteristics are distinct from those of the renal scanning agent ^{99m}Tc(III)-DMSA, as shown by the negligible renal retention. On the other hand it exhibits selective tumor-seeking properties and clinical screening has demonstrated its usefulness in the detection and imaging of medullary thyroid carcinoma and head and neck tumors, in particular squamous carcinoma and rhabdomyosarcoma [97-103]. Convenient methods for the preparation of this interesting ^{99m}Tc-labeled tumor agent have been reported [104,105].

c). COMPLEXES OF ^{99m}Tc WITH POLYPEPTIDES

Polypeptides are able to bind Tc by the presence of amide nitrogens, carboxyl oxygens and in most cases also thiolates as ligand atoms. One of the newer tracer agents, ^{99m}Tc-aprotinin, was found to show some peculiar features and clinical usefulness. Aprotinin is a polypeptide of 58 amino acids (M.W. ~6500) including 8 cysteine moieties. It is not clear whether the cysteinyl sulfhydryl groups take part in the complexation of Tc, as they are arranged in 4 pairs of disulfide bridges [109-110]. The renal uptake of ^{99m}Tc-aprotinin in humans is over 50% after 1 hr and can reach up to 68% after 3 hr and remains stable between the second and eight hour after injection [8,111]. Compared to ^{99m}Tc-DMSA this new kidney imaging agent shows a faster blood clearance, reduced urinary excretion (2.7% of injected dose during the first 2 hr) and higher renal fixation, which compensates for the slightly higher liver activity [112]. It is much less sensitive to high serum creatinine levels than chlormerodrin or ^{99m}Tc-DMSA [8] and allows detection of residual functioning parenchyma also in severe kidney failure. The pathway for renal fixation of ^{99m}Tc-aprotinin is thought to be glomerular filtration as a light-chain protein followed by reabsorption and retention by the tubular cells [113], but evidence for this has not been reported.

2. CURRENT STATUS AND PERSPECTIVES OF MORPHOLOGICAL IMAGING AGENTS

Radiopharmaceuticals currently used for static renal imaging are all ^{99m}Tc-radiopharmaceuticals. The renal retention of ^{99m}Tc-gluconate and glucoheptonate is markedly lower than of the other agents, but due to the fast urinary excretion of the non-retained fraction and a negligible liver uptake the images are of excellent quality with sharp contrast and low body-background. ^{99m}Tc-DMSA is the most intensively used agent, although it suffers from the fact that imaging can start only 3 to 6 hours after injection. More clinical information is required to evaluate whether ^{99m}Tc-aprotinin has objective superiority over ^{99m}Tc-DMSA. A comparison of the renal uptake kinetics of ^{99m}Tc-glucoheptonate, ^{99m}Tc-DMSA and ^{99m}Tc-aprotinin can be made from Figure 3, showing their renograms in a baboon.

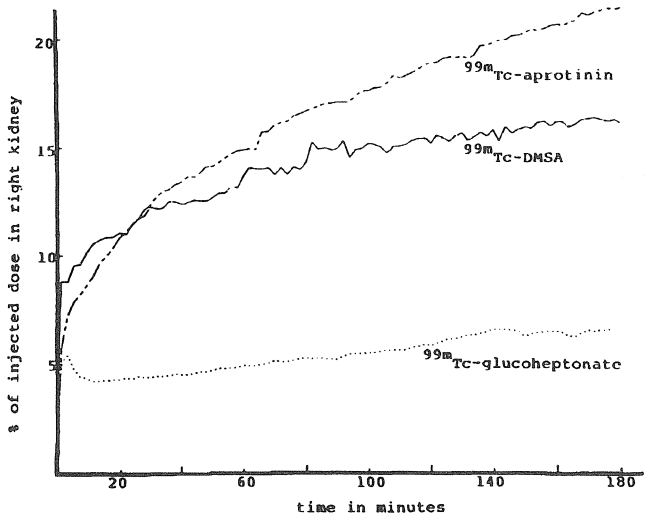


Figure 3. Paired study of ^{99m}Tc-DMSA, ^{99m}Tc-glucoheptonate and ^{99m}Tc-aprotinin in a baboon. Each renogram shows the uptake of the indicated tracer agent in the right kidney of the baboon as a function of time after injection (Verbruggen, A.M., and Borman, G., unpublished results).

A real improvement could still be provided by an agent that is taken up in the kidneys to a high degree (70-90%) within 30-60 min after injection without liver or residual blood activity.

B. Radiopharmaceuticals for the Determination of Glomerular Filtration Rate

The measurement of glomerular filtration rate (GFR) is a standard technique for evaluating renal function. The requirements for a compound to be suitable for the measurement of GFR have been listed in many review papers [e.g. 3,4,6,8,122] together with a detailed discussion on the radiopharmaceuticals used or proposed for this purpose. Ideally such compounds are polar, do not diffuse out of the plasma to the extracellular fluid and are cleared completely from the blood by the process of glomerular filtration without reabsorption, fixation or secretion at the tubules. This implicates also that they should not be or are only loosely bound to plasma proteins and are not metabolized in the plasma or kidneys. The compound should be non-toxic and must not influence renal function over a wide range of concentrations

As indicated in Table 1, GFR can be calculated from the concentration of such an agent in urine and plasma and the urine production rate. Radiopharmaceuticals labeled with a gamma-emitting radionuclide can be quantitated easily and accurately in both plasma and urine and can thus be very appropriate for this purpose. Their use has become still more attractive as techniques have been developed for quantitating total and individual renal function using external detection without the need for blood sampling [123-125].

1. INULIN AND DERIVATIVES

The reference method for determination of GFR has for many years been the measurement of the clearance of inactive inulin. It is, however, not ideal for routine clinical work due to the need for constant intravenous infusion and accurate urine sampling and a somewhat cumbersome analytical determination [6]. To improve the practical use of inulin, a number of radioiodinated derivatives have been proposed [126-128]. However, they suffer from dissociation of the radioiodine in vitro and in vivo, leading to erroneous GFR values due to the markedly lower renal clearance of inorganic iodine [122]. ^{51}Cr-inulin is probably the most appropriate derivative [129], but the detection and radiation characteristics are far inferior to those of technetium-99m labeled compounds.

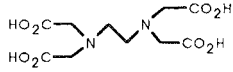
2. RADIOIODINATED CONTRAST AGENTS

Radioiodinated analogues of the urological contrast media diatrizoate and iothalamate have been studied extensively for renal function testing. Clearance values of ^{131}I-diatrizoate were however low because of release of radioiodine proportional to the specific activity of the preparation [6,130]. Although generally a significant correlation has been found between the clearances of diatrizoate and inulin, the extrarenal pathway of diatrizoate becomes more important in severe renal failure [131].

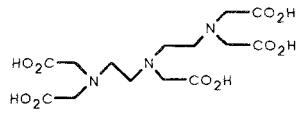
Radioiodinated iothalamate differs chemically only slightly from diatrizoate but it is more stable with respect to the release of free iodide [132]. Its renal clearance was found to correlate closely with inulin clearance in a large number of patients [122] and is now accepted as a standard [131] against which other potential agents are validated. The unfavorable radiation dosimetry of radioiodinated compounds and their limited stability is however a restriction to their routine clinical use.

3. METAL CHELATES

Complexes of transition metals with ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA) are very stable and also very polar due to the presence of 4 or 5 carboxylic groups and 2 or 3 amino groups (Figure 4). The observation of the rapid renal excretion of ^{14}C-EDTA [133] and ^{14}C-DTPA [134] in humans lead to the study of a large number of physiologically stable chelates of EDTA and DTPA with a variety of radionuclides including ^{51}Cr, ^{58}Co, ^{99m}Tc, ^{111}In, ^{113m}In, ^{140}La, ^{169}Yb [3]. For practicial reasons of availability, convenience and dosimetry only ^{51}Cr-EDTA and ^{99m}Tc-DTPA have gained wide acceptance and routine clinical use.



EDTA



DTPA

Figure 4. Structure of EDTA and DTPA.

The complex of chromium with EDTA is one of the most stable metal-EDTA complexes, with a formation constant of 10^{24} [135]. On this basis one can assume that ligand exchange with strongly complexing proteins, e.g. metallothionein, is absent or extremely low. Moreover it seems that complexes based on ligands with multiple carboxylic groups are not reabsorbed in the proximal tubules, as is also the case for ^{99m}Tc-citrate [37]. Due to its low protein binding (<2%), the lack of tubular secretion [136] and minimal extrarenal elimination, ^{51}Cr-EDTA has had extensive clinical application for GFR determination using both single-injection and continuous infusion techniques. Many authors have compared ^{51}Cr-EDTA and inulin clearance and a very close correlation has been found [e.g. 137-140]. As a rule ^{51}Cr-EDTA values are slightly lower. However, in a study using a gamma camera technique without collection of blood or urine Rehling et al. concluded that

single kidney GFR calculated from the plasma clearance of ^{51}Cr-EDTA overestimated the renal clearance of inulin on an average by 11.3% [141].

^{99m}Tc-DTPA was found to be more suitable for renal function studies than ^{99m}Tc-EDTA because of lower protein binding and higher stability [142]. However, values reported for its stability constant range from 10^{17} to 10^{26} [142-144], which lets us suppose that complexes with different structure were involved in the respective determinations. The use of ^{99m}Tc DTPA for GFR determination was introduced in 1972 by Klopper et al. [145]. During studies in dogs using constant infusion techniques they observed no change in ^{99m}Tc-DTPA clearance at different urine flow rates or following tubular blockade with probenecid. Clearance rates calculated from plasma levels in patients underestimated GFR by about 8% as compared with ^{125}I-iothalamate, but the authors ascribed this partly to protein binding and impurities in the preparation. Hosain [146] and Carlsen et al. [147] observed that GFR-values obtained with ^{99m}Tc-DTPA prepared from some home-made or some commercial kits correlated well with results obtained with ^{111}In-DTPA or ^{51}Cr-EDTA, but a clear underestimation was found using other kits. These findings were confirmed by Russell et al. [148-149] who concluded that the error in GFR is due to protein binding, presumably provoked by an impurity. In order to eliminate this error they suggest measuring the protein binding separately for each patient or to use preparations with an impurity level under 1%, a standard that can be met in practice even over a wide variety of daily work conditions [150]. Recent studies with ^{99m}Tc-DTPA prepared from commercial kits have demonstrated the absence of clinical relevant differences between the plasma clearance of ^{99m}Tc-DTPA after a bolus injection and the renal clearance of inulin and ^{51}Cr-EDTA [140,141,151].

Because of the far more favorable imaging and dosimetry characteristics of ^{99m}Tc, the continuous availability and the ease of preparation, ^{99m}Tc-DTPA is currently the agent of choice for radionuclide imaging clearance

studies. A variety of simplified methods for rapid measurement of GFR using ^{99m}Tc-DTPA with minimal inconvenience for the patient have been proposed. A comparative evaluation of them can be found in recent papers [152-154]. The requirement of using reliable quality control procedures to eliminate impure preparations has however to be emphasized. The rapid renal excretion of ^{99m}Tc-DTPA and its initial high renal uptake permit both static and dynamic imaging studies and a number of additional renal parameters can be assessed with this widely used renal radiopharmaceutical [155-157].

C. Radiopharmaceuticals for Measurement of Effective Renal Plasma Flow

Renal blood flow (RBF) can be determined by measuring the amount of a substance extracted from the blood by the kidneys per unit of time and dividing this value by the difference in renal arterial and venous concentration for this particular agent. If the amount of this compound in the blood cells is unaltered during passage through the kidney, renal plasma flow (RPF) can be calculated more easily from the amount excreted in the urine per unit of time divided by the renal arteriovenous concentration difference. The determination would be simplified by using a compound extracted from the blood completely during a single circulation through the kidney, as the renal venous concentration is then zero and the peripheral venous plasma concentration can be used instead of renal arterial plasma level. Values obtained in this way are called effective renal plasma flow (ERPF) to indicate that renal venous plasma concentration was not measured. ERPF is always lower than the actual RPF, as no compound is known with an extraction efficiency of 100% and not all renal blood passes through nephrons. The error can however be negligible by using a substance that is extracted nearly quantitatively or can be overcome by correcting for the lower extraction efficiency if this is constant over a wide range of physiological conditions.

A high extraction efficiency requires that a compound is removed from the plasma not only by glomerular filtration - this process can clear at most nearly 20% of the renal blood - but also and mainly by tubular secretion. The nonradioactive standard for ERPF measurement is para-aminohippuric acid (PAH) (Figure 5). During one renal passage about 90% of PAH is excreted into the urine, partially (~20%) by glomerular filtration and for the rest (~80%) through tubular secretion [3,4,122]. It is handled in the tubules by the hippurate organic anion pathway and its renal behavior is characterized by a clear transport maximum. Its tubular secretion is inhibited by previous administration of compounds competitive for the same transport system, such as probenecid, phenol red and penicillin G [158].

The structural requirements for efficient secretion by the renal tubules have been studied in detail for organic anions by comparing the renal handling of a large number of carboxylic acids, mercurial diuretics, sulfonic acids and sulfonamides. Although no clear definition of substrate specificity could be put forward, Despopoulos [159] postulated for hippurate-like substances a three-point interaction between the substrate and receptor. Two oxygens of the carboxyl group would react with appropriate complementary loci on the receptor to form an ionic bond and the oxygen of the carbonyl group would provide a supporting hydrogen bond. He also concluded that the presence of an aliphatic chain with a structure as shown

$$R\text{-}CO\text{-}NX\text{-}(CH_2)_n\text{-}COO^-$$

$$X = H \text{ or } CH_3,$$
$$n = 1 \text{ to } 5$$

or a reactive group arrangement that mimics the binding properties of this chain in its folded or fully extended state renders molecules a candidate for secretion by the renal hippurate transport system. The carbonylglycine (-CO-

NH-CH_2-COO^-) side chain of PAH is the simplest and most polar example of such a chain. More recent studies on various structural categories of secreted anions which do not correspond to these requirements (e.g. urate, sulfisoxazole, acetylsalicylate, chenodeoxycholate), has led to the concept of multiple renal secretory systems for organic anions [160]. Most radiopharmaceuticals proposed for ERPF studies can be regarded, however, as substances with a hippurate-like behavior.

1. SUBSTANCES RELATED TO HIPPURIC ACID

In view of the convenience and accuracy of radioactivity measurements in urine and plasma it was logical to develop radiolabeled analogs of PAH. As none of the atoms of PAH (C,H,O,N) can be replaced by a suitable gamma-emitting radioisotope, Tubis et al. [161] developed in 1960 a radioiodinated derivative, ^{131}I-o-iodohippurate, commonly called hippuran or OIH (Figure 5). It has been the standard radiopharmaceutical for radionuclide ERPF measurements since then [4]. For reasons of more favorable radiation characteristics with respect to both gamma camera imaging and dosimetry the iodine-123 analogue was later prepared [e.g. 162,163] and has gradually replaced ^{131}I-OIH. Hippuran differs from PAH by the substitution of a polar amino group in the para position by a lipophilic iodine atom in the ortho position. The ratio of hippuran clearance to PAH clearance was reported to be 0.9 in comparative ERPF studies in humans [164,165] whereas other authors found values in the range 0.8-0.9 [166-168]. The lower value for OIH can probably be attributed to its diffusion into the red cells and its higher protein binding as compared to PAH [168], which in turn is probably due to the lower polarity. As is the case with the majority of radioiodinated agents, hippuran suffers from instability due to the release of free iodide. A consequence of this decomposition is the gradual decrease of extraction efficiency of OIH with time: this changes from 88% immediately after injection, when mainly OIH is

present, to as low as 50% at 1 hr, when OIH is greatly excreted and iodide and possibly small quantities of iodobenzoic acid remain in the circulation [169].

Despite the wide-spread use of ^{131}I-labelled hippuran during the last 25 years, it is not an ideal radiopharmaceutical. The radiation dose of ^{131}I-OIH is relatively high due to the emission of β–radiation and the 8 day physical half-life of the radionuclide. Only small doses can be administered resulting in poor imaging characteristics for the 364 keV c-radiation. These problems can greatly be overcome by the use of ^{123}I-OIH that is readily available in some countries, but it will never have "off-the-shelf-availability" due to the high cost of the cyclotron produced radioisotope and its short half-life.

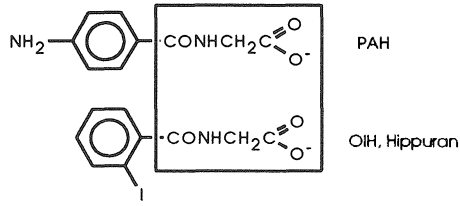


Figure 5. Structure of para-aminohippuric acid (PAH) and o-iodohippuric acid (hippuran, OIH) with indication of the supposed essential moieties for interaction with the tubular transport system.

Radiobrominated analogues of hippuran, labeled with ^{77}Br or ^{82}Br, showed in vivo properties analogous to iodohippurate in mice and rabbits but appeared to be not useful for renal function studies in human volunteers [170,171]. A ^{75}Se-labeled derivative of hippuric acid showed a biological behavior similar to ^{131}I-OIH in rabbits [172].

A completely different approach to obtain a suitable hippuran derivative was proposed by Wenzel et al. [173]. They substituted the aromatic nucleus of OIH by ruthenocene labeled with ^{97}Ru, a radioisotope with suitable nuclear properties for patient studies. In rabbits the plasma clearance of this so-called ruppuran (Figure 6) was slightly higher than that of ^{125}I-OIH.

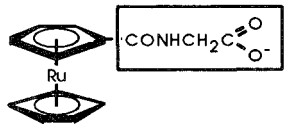
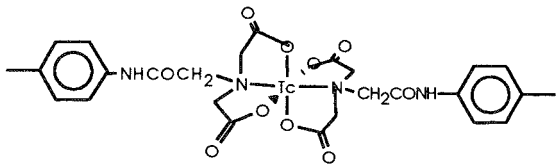


Figure 6. Structure of ruthenocenoylglycine (ruppuran).

This indicates that in hippurate-like substances the carbonylglycine side chain is of more importance for efficient tubular secretion than the phenyl nucleus. Studies in humans have not been reported with this interesting tracer agent.

Chervu et al. [174] coupled PAH with nitrilotriacetic acid via the aromatic amino group to obtain PAHIDA, a derivative that easily can be labeled with ^{99m}Tc.



R = -CO-NH-CH_2-COOH

Figure 7. Proposed structure of Tc-PAHIDA.

In mice and rats urinary excretion of ^{99m}Tc-PAHIDA is similar to that of ^{131}I-OIH. In dogs however, the renal clearances of ^{99m}Tc-DTPA and ^{99m}Tc-PAHIDA are not significantly different and the rate of clearance is not decreased by treatment with probenecid [175], which indicates the absence of tubular secretion. This has been confirmed by Verbruggen et al. [176] who found in a paired study on 5 volunteers plasma clearances of 99.4 ñ 5.7, 96.8 ñ 8.4 and 516.1 ñ 25.1 ml/min/1.73m^2 for respectively ^{99m}Tc-PAHIDA, ^{99m}Tc-DTPA and ^{131}I-OIH. It can be assumed that the technetium atom in ^{99m}Tc-PAHIDA is bound between two ligand molecules, in a similar way as it has been shown for the structurally similar ^{99m}Tc-HIDA agents [177,178]. Despite the presence of two hippurate moieties this complex is apparently so

different from PAH that it can no longer interact efficiently with the renal tubular carrier mechanism

2. ^{99m}Tc THIODIGLYCOLIC ACID (^{99m}Tc-TDG)

A few reports have described the potential use of ^{99m}Tc-TDG as a replacement for radioiodinated hippuran [179-181]. Labeling of thiodiglycolic acid (HOOC-CH_2-S-CH_2-COOH) with ^{99m}Tc using tin metal as reductant results in the formation of 2 complexes, the relative amount of which can be altered by heating. The first compound showed in rats renal clearance similar to ^{99m}Tc-DTPA, while the other showed a behavior similar to OIH. In humans the renal clearance of complex 2 was 1.88 times that of ^{99m}Tc-DTPA, but only 41% of OIH clearance. This indicates excretion at least partially by tubular secretion but with a low extraction efficiency. It was concluded that ^{99m}Tc-TDG cannot be used for direct determination of ERPF.

3. ^{99m}Tc-DADS AND DERIVATIVES

Davison and coworkers introduced in 1979 an original chelating structure, based on thiolate and amide nitrogen donor atoms [182,183]. They reported that ^{99m}Tc-DADS, a complex of ^{99m}Tc with N,N'-bis (mercaptoacetyl) ethylenediamine (a diamide disulfur ligand) is rapidly excreted by the kidneys, like OIH. The rapid urinary clearance of this complex was confirmed in mice, rats and rabbits [184,185] and in humans [186]. Studies in mice in the presence of the renal tubular transport inhibitors probenecid and 2,4-dinitrophenol confirmed active secretion by tubular cells [187]. However, ^{99m}Tc-DADS was still clearly inferior to radioiodinated hippuran due to partial hepatobiliary excretion and slower renal excretion. The inhibitory effect of probenecid on the renal excretion of ^{99m}Tc-DADS supports carrier mediated handling by the hippurate transport system. No carbonylglycine-like moiety is present in the

complex but it can be assumed that the [-CO-N-Tc=0) arrangement is able to mimic the binding properties of the hippurate side chain (figure 8).

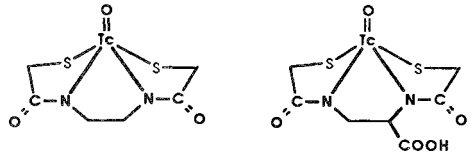


Figure 8. Structure of Tc-DADS and Tc-CO_2DADS.

To improve the biological characteristics of ^{99m}Tc-DADS several groups synthesized and evaluated a high number of derivatives [188-194]. The compound with a carboxyl group on the ethylene bridge between the two amide nitrogens, called CO_2DADS, bears in its structure a similar carbonylglycine sequence as the hippurate side-chain and its ^{99m}Tc complex was found to be the most promising ^{99m}Tc labeled substitute for OIH so far known. Complexation of Tc-99m with this new chelating agent results in two isomeric components, separable by HPLC, with the carboxyl substituent respectively syn or anti with respect to the [Tc = O] group. Component A, first eluted during HPLC, is the syn-isomer [195] and appeared comparable to OIH in animals, although pretreatment with probenecid caused a more pronounced inhibition than for OIH. Component B however was cleared more slowly and accumulated to a higher degree in the liver.

Clinical evaluation of ^{99m}Tc-CO_2DADS-A in human volunteers and patients [196,197] showed that it was clearly superior to ^{99m}Tc-DTPA with a higher kidney-to-background ratio and faster urinary excretion without hepatobiliary excretion. Compared to OIH, the urinary excretion was slightly less and decreasing renal function impaired excretion of ^{99m}Tc-CO_2DADS-A more than that of OIH. These results were however not confirmed in studies on kidney-transplant patients. In case of acute graft rejection, Bubeck et al. [198] did not observe the typical diagnostically useful accumulation curve which

results from high retention in the kidney parenchyma. The plasma clearances obtained for ^{99m}Tc-CO_2DADS-A in nine patients were on the average only 36% of those of ^{131}I-OIH and it was concluded that this ^{99m}Tc-tracer agent is not suitable as a substitute for hippuran.

Verbruggen and coworkers [199,200] demonstrated that the two epimers of ^{99m}Tc-CO_2DADS, reported and tested by other authors, are each actually a mixture of two enantiomers, a D- and L-form. They separated the four stereoisomers and found that isomer DA (the isomer which has a syn configuration and is eluted first on HPLC of the reaction mixture after labeling of D-CO_2DADS with ^{99m}Tc) shows clearly superior renal excretion characteristics in mice and a baboon with respect to both racemic ^{99m}Tc-CO_2DADS-A and the three other isomers.

Isomer LA, also with a syn configuration, is only slightly inferior to isomer DA, but in mice its liver uptake is higher and in baboon the plasma clearance is lower. This higher retention in liver and blood can be ascribed to the fact that compounds with a "natural" L-configuration interact with and bind to biological systems more easily than do D-compounds. Isomers DB and LB, both bearing the carboxyl group anti to the TcO-core, show a slow urinary excretion in mice and a low plasma clearance in the baboon. Liver uptake of isomer LB in mice is pronounced (Table 3).

Table 3. Biodistribution of the Four Stereoisomers of ^{99m}Tc-Co_2DADS

MOUSE

Isomer	Configuration	% Injected Dose at 10 min p.i.		
		Urine	Kidneys	Liver
*D*A	syn	84.3	3.4	3.0
*L*A	syn	75.7	3.5	6.3
*D*B	anti	73.3	4.0	6.2
*L*B	anti	56.7	3.6	27.5
^{131}I-OIH	---	78.8	3.0	1.8

Table 3 (cont'd)

BABOON

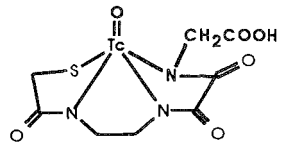
Isomer	Configuration	1-Hr Plasma Clearance (% OIH Value)	Liver Uptake (% of I.D.) (40-45 min p.i.)
*D*A	syn	44.9	0.9
*L*A	syn	32.0	0.1
*D*B	anti	16.4	2.8
*L*B	anti	11.7	3.4

^{99m}Tc-CO_2-DADS contains in its structure a carbonylglycine moiety (Figure 8) and the improved renal handling, as compared to ^{99m}Tc-DADS, could be explained on this basis. However, from the results with the four stereoisomers it appears that a syn configuration is essential for optimal renal handling. It is thus more likely to suppose that the structural requirements for efficient three-point interaction with the tubular transport receptor are met by the oxotechnetium-glycine (TcO-N-CH-COO^-) sequence and not so much by the carbonylglycine moiety. The TcO-oxygen would then replace the amide-oxygen of Despopoulos' theory and the rest of the CO_2-DADS backbone would take the place of the aromatic nucleus in OIH. Only in syn complexes are the oxotechnetium and carboxyl group oriented in the same direction and able to bind simultaneously to the respective binding sites of the tubular transport receptor.

Clinical evaluation of ^{99m}Tc-CO_2DADS-DA in 6 volunteers revealed a mean plasma clearance of 58% of the value obtained for co-injected OIH and a 1 hr-urinary excretion slightly higher than that of OIH. Hepatobiliary excretion was never observed [201]. These results suggest a potential clinical use of ^{99m}Tc-CO_2DADS-DA as a substitute for radioiodinated hippuran, but the need for an HPLC purification step during the preparation discouraged further development.

4. ^{99m}Tc-MERCAPTOACETYLTRIGLYCINE

To obviate the formation of stereoisomers and the need for their preparative separation, Fritzberg and coworkers [38,39,202] modified the diamide dithiolate (N_2S_2) structure of CO_2DADS to a triamide mercaptide (N_3S) ligand. This allows the presence of a carboxylate group without the introduction of an asymmetric carbon atom. One of the simplest members of this series, bearing an acetate group on the third amide nitrogen, is mercaptoacetyltriglycine or MAG_3. Labeling with technetium-99m results in a radiochemical product with a square pyramidal structure similar to that of ^{99m}Tc-CO_2DADS [204]. On biological evaluation in normal and probenecid treated rats ^{99m}Tc-MAG_3 (Figures 9-10) was found to be superior to ^{131}I-OIH, as indicated by a faster urinary excretion rate, a higher plasma clearance and a more favorable extraction efficiency, which clearly indicates renal excretion by active tubular transport. On the other hand Muller-Suur et al. [204] reported a higher extrarenal clearance presumably due to bile excretion, even after HPLC purification of the labelled reaction mixture.



Tc-MAG_3

Figure 9. Structure of Tc-MAG_3

Based on the animal results ^{99m}Tc-MAG_3 has been evaluated in volunteers [205] and now also in a wide population of patients [206-217]. The blood clearance initially found in sequential studies with ^{131}I-OIH was nearly 150% of that of OIH, but later these results could not be reproduced [205]. The ratio of ^{99m}Tc-MAG_3 to OIH plasma clearance has now been accepted to be

in the range of 0.61-0.65 [209,210], mainly due to the smaller volume of distribution of ^{99m}Tc- MAG_3 (65% of OIH value). This in turn is attributed to the higher degree of protein binding : 86-91% for the ^{99m}Tc compound versus 69-76% for OIH in paired studies [205,211]. ^{99m}Tc-MAG_3 images are unanimously judged superior to those of ^{131}I-OIH and of equal quality compared to ^{123}I-OIH, regardless of renal function state. There was however evidence for some hepatobiliary excretion of MAG_3 to a degree not exceeding 4% [209]. The relative renal functions determined with ^{99m}Tc-MAG_3 are highly correlated to those of OIH studies in patients with renal disorders and the renograms exhibit qualitatively the same curve-shapes in normal volunteers and patients with acute graft rejection or acute tubular necrosis. From the acquired experience it is generally accepted that ^{99m}Tc-MAG_3 is a suitable replacement for ^{131}I-and ^{123}I-hippuran in routine renal imaging, relative function and transit time studies and also for the determination of the effective renal plasma flow [215,216]. It is also suggested that a single ^{99m}Tc-MAG_3 examination can replace both the perfusion study with ^{99m}Tc-DTPA and the function study with ^{131}I-OIH and in some cases even static renal imaging using ^{99m}Tc-DMSA. A kit for the preparation of ^{99m}Tc-MAG_3 is available. The initial labeling kits had a shelf-life after reconstitution of only one hour due to the gradual increase of side-products with a high hepatobiliary uptake. To overcome this inconvenience, Solanki et al. have proposed storage of the reconstituted preparation in frozen unit doses [218]. The problem has now been solved by modification of the labeling kits [219]. ^{99m}Tc-MAG_3 from a kit has been compared with HPLC- purified ^{99m}Tc-MAG_3 in animals and no significant differences were observed between the two preparations [220]. Figure 10 shows the renograms obtained in the same baboon with respectively ^{99m}Tc-MAG_3, ^{99m}Tc-DTPA and ^{123}I-hippuran.

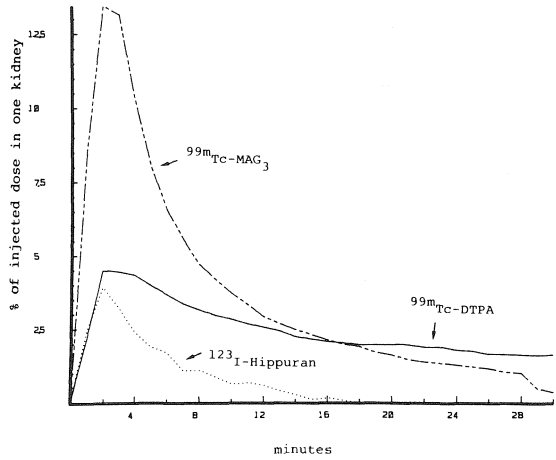


Figure 10. Paired study of ^{99m}Tc-MAG3, ^{99m}Tc-DTPA and ^{123}I-Hippuran in a baboon. Each renogram shows the uptake of the indicated radiopharmaceutical in the right kidney of the animal in function of time after injection (Verbruggen, A.M. and Bormans, G., unpublished results).

Due to its square pyramidal structure ^{99m}Tc-MAG$_3$ can exist in two enantiomeric forms with different orientation of the oxotechnetium group with respect to the N_3S core (Figure 11).

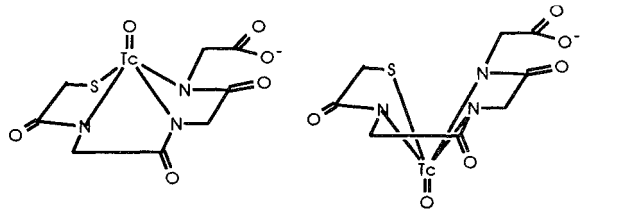


Figure 11. Representation of the Structure of 2 Enantiomers of ^{99m}Tc-MAG$_3$

Verbruggen et al. separated these isomers by an indirect method and studied their biodistribution in a volunteer [221], but no significant differences were found with respect to plasma clearance, rate of urinary excretion, shape of the renograms and uptake in the liver (Table 4).

Table 4. Comparison of the Biological Properties of the Enantiomers of ^{99m}Tc-MAG$_3$ in a volunteer [221]

	^{99m}Tc-MAG$_3$ (Mixture of Isomers)	Isomer A	Isomer B
Plasma Clearance as % of OIH value	61.8	65.9	61.5
Urinary Excretion as % of OIH value			
0-35 min	93.6	96.1	96.4
0-65 min	99.4	100.8	101.7
Uptake in Liver at 40-45 min p.i. (% of I.D.)	4.0	4.95	2.97

^{99m}Tc-MAG$_3$ contains also the oxotechnetium-glycine sequence, but in this complex the terminal carboxyl group can always be oriented in the same direction (syn) as the oxotechnetium core due to its free rotation. The identical handling of both isomers by the highly stereospecific tubular transport system can be explained on this basis and this constitutes additional support for the hypothesis that the oxotechnetium-glycine and not the carbonylglycine moiety is the essential structural part which makes ^{99m}Tc-MAG$_3$ a suitable substitute for OIH.

5. DERIVATIVES OF ^{99m}Tc-MAG$_3$: ^{99m}Tc-MAGAG

Derivatives of MAG$_3$ with an asymmetric carbon atom form ^{99m}Tc diastereoisomers which can easily be separated by reversed-phase HPLC. Verbruggen et al. [222-224] prepared 8 optically pure (D or L) methyl derivatives of MAG$_3$ and isolated, after labeling with ^{99m}Tc, the 16 different stereoisomers. The most remarkable results were found in the four isomers of ^{99m}Tc-mercaptoacetylglycylalanylglycine (^{99m}Tc-MAGAG). Their structures are shown in Figure 12.

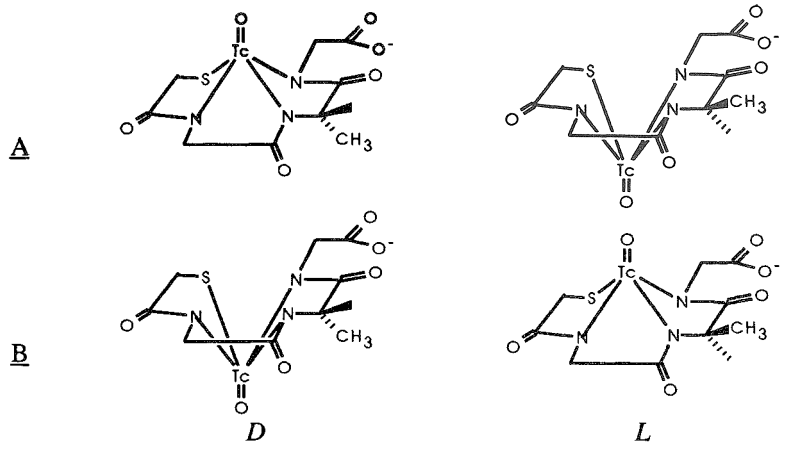


Figure 12. Representation of the structure of the 4 diastereoisomers of ^{99m}Tc-MAGAG. As the absolute configuration has not yet been determined, the enantiomers are arbitrarily named A and B [225].

As their absolute configuration has not yet been elucidated, designation is by configuration of the alanyl moiety (D or L) and order of elution upon HPLC separation (A or B). In mice ^{99m}Tc-MAGAG-DA and to a lesser extent isomer DB are superior to ^{99m}Tc-MAG_3 in terms of faster urinary excretion, lower retention in the kidneys and lower liver uptake (Table 5).

Table 5. Biodistribution of the Isomers of ^{99m}Tc-MAGAG in Mice. (% of Injected Dose in Organ at 10 Min.)

	^{99m}Tc-MAG_3	^{99m}Tc-MAGAG			
		DA	DB	LA	LB
Urine	66.9	74.5	68.2	55.8	7.4
Kidneys	12.9	6.4	12.2	5.4	86.6
Liver	6.9	4.3	5.8	28.0	8.1
n	24	16	16	6	12

On the other hand, isomer LB, differing from DA only by the configuration at the asymmetric carbon atom, was found to accumulate for more than 85% in the kidneys from 10-20 min after injection, the highest value of renal uptake ever reported (Figure 13). The LA-isomer is characterized by significant liver uptake. The faster urinary excretion of the D-isomers agrees well with the already mentioned principle that compounds with an L configuration are reabsorbed in the proximal tubule more efficiently than the D isomers [16]. On the other hand, the selective affinity of biological systems such as receptors and transport carriers for compounds with an L-configuration can explain the uptake and/or binding of isomers LA and LB in respectively the liver and the kidneys. As the terminal carboxylate of ^{99m}Tc-MAGAG can rotate, the different biological behavior of the four stereomers of ^{99m}Tc-MAGAG must be explained on the basis of the presence of an asymmetric carbon atom and the relative position of the methyl substituent with respect to the oxotechnetium and carboxyl group. The influence of the D or L configuration on uptake and/or retention in the liver or kidneys is described above. In isomer DA the methyl substituent apparently does not interfere with an efficient interaction between the tracer agent and the hippurate transport receptor. However, the in vivo results seem to indicate that the reverse is true for isomer DB. If in Figure 12 isomer DB is turned so that the oxotechnetium group points also to the top of the figure (as in isomer DA), then the carboxylate and oxotechnetium group can still be oriented syn, but the methyl substituent is now moved to the left of the molecule, contrary to the situation in isomer DA. The results of the biodistribution indicate that such a conformation does not fit efficiently with the renal tubular transport system.

Evaluation of the isomers DA and DB in a baboon [225-226] showed that isomer DA exhibits superior renal excretion characteristics. Its

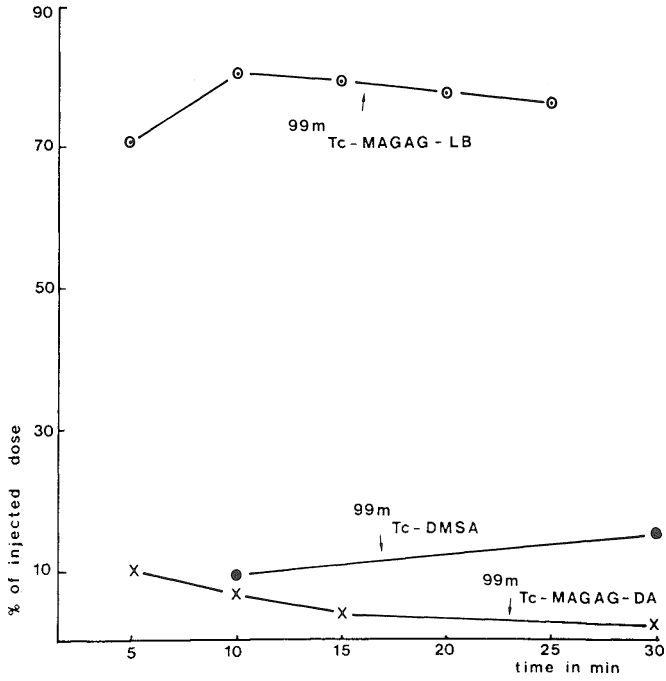


Figure 13. Renal uptake in mice of isomers DA and DB of ^{99m}Tc-MAGAG and of ^{99m}Tc-DMSA [228].

renogram is characterized by a shorter time to peak, a higher renal uptake at peak maximum, a steeper renal excretion curve and a lower retention in the kidneys after 30 min. Isomer DB resembles more ^{99m}Tc-DTPA with a clearly longer renal retention (Figure 14).

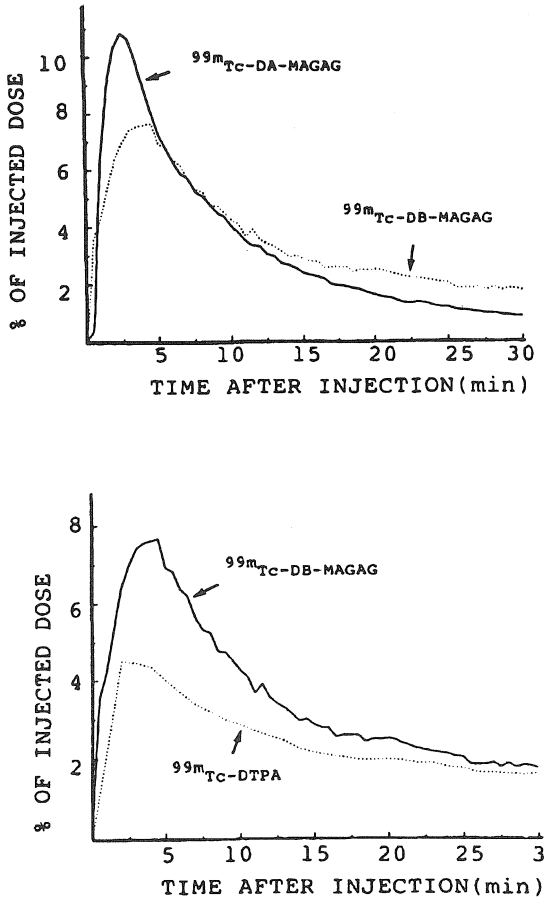


Figure 14. Comparison of the renograms of ^{99m}Tc-MAGAG DA, DB and ^{99m}Tc-DTPA in a baboon [225].

In a paired study on six human volunteers [227] it was shown clearly that ^{99m}Tc-MAGAG-DA approaches OIH more closely than ^{99m}Tc-MAG_3 (Table 6), and this derivative of ^{99m}Tc-MAG_3 is without doubt the best technetium-99m labeled substitute for OIH reported up to now. Again, however, the need for an HPLC-purification step is an obstacle to its widespread use and ^{99m}Tc-MAG_3 remains at this moment the agent of choice for ERPF studies.

Table 6. Comparison of the Renal Excretion Characteristics of ^{99m}Tc-MAG$_3$ and ^{99m}Tc-MAGAG-DA in 6 Volunteers [227].

	^{99m}Tc-MAG$_3$	^{99m}Tc-MAGAG-DA
Time to renal maximum (min)	3.67 ± 1.12	2.96 ± 0.60
Activity/kidney at max. (% of Injected Dose)	10.35 ± 2.11	8.60 ± 0.98
Time to half the max. after peak (min)	3.03 ± 0.26	2.86 ± 0.37
Renal retention at 30 min as % of renal max.	8.21 ± 0.93	2.22 ± 1.06
Plasma clearance as % of hippuran value	64.11 ± 4.17	78.50 ± 4.35
Distribution volume as % of hippuran value	63.22 ± 3.17	68.34 ± 3.52
Urinary excretion as % of hippuran value		
0-35 min	97.98 ± 1.08	104.50 ± 3.38
0-65 min	101.56 ± 0.94	105.40 ± 3.44

IV. SUMMARY

The extreme sensitivity of the biological characteristics of some of these complexes to their stereochemistry suggests that one or more specific sites are involved. Whereas in this case, these sites are probably part of the transport processes the phenomena described do suggest that similar small metal complexes can interact with topographically restrained sites in vivo and may in the future be used as probes for such sites (rather than as devices for imaging excretion).

ACKNOWLEDGEMENTS

The authors wish to thank Dr. P. Devos for his critical review of the draft copy and B. Cleynhens for help in preparing the figures.

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