SPECIFICITY IN DRUG-PROTEIN INTERACTION

thermodynamic, kinetic and stereochemical aspects
SPECIFICITY IN DRUG PROTEIN INTERACTION
THERMODYNAMIC, KINETIC AND STEREOCHEMICAL ASPECTS

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CONTENTS

General Introduction 1

SECTION I

SPECIFICITY IN HYDROPHOBIC INTERACTIONS

Thin-layer chromatographic separation of free fatty acids 10
analysis and purification of radioactively labelled fatty acids

The extent of hydrophobic binding area 17
studied by fatty acid binding to albumin

SECTION II

SPECIFICITY STUDIED BY NUCLEAR MAGNETIC RESONANCE

Non-exponential relaxation of the methyl protons of acetrizoate
in solution 45

A nmr study of the kinetics of the binding of the renal contrast
medium acetrizoate to albumin 55

The kinetics of the binding of acetrizoate to albumin in relation
to its renal clearance 81

SECTION III

SPECIFICITY IN DIASTEREOMERIC INTERACTIONS

Stereoselectivity and affinity in molecular pharmacology 88
1. The correlation of stereoselectivity and activity

Stereoselectivity and affinity in molecular pharmacology 125
2. A molecular basis for eudismic-affinity correlations

Summary 142

Samenvatting 145

Curriculum vitae 149
phase seems, therefore, most suitable to eliminate unwanted side effects. Studies of structure-activity relationship both for the desired and for the unwanted effects provide means to find out whether particular structural changes lead to a separation of these effects. Such a separation is principally only possible if the types of receptors involved, in main and side effect, are different. In this case high activities will in general contribute to selectivity in action, since high activity usually will be based on a high degree of complementarity between drug and receptor. A high degree of complementarity towards different types of receptors is unlikely.

As a matter of fact in the discussion of structure-activity relationship, the concept of a drug-receptor interaction is at the background. The part-processes involved in the drug-receptor interaction as far as the role of the various groups in the bioactive molecule in the interaction is concerned are not taken into account.

While structure-activity relationship studies mainly are an approach on the molecular level, structure-action relationship studies are an approach primarily on the submolecular level. In this latter approach the interaction of the drug molecule with its active site is analysed in terms of the contributions of the various groups of the drug molecule. Moreover, the spatial demands of the receptor with respect to the different groups in the pharmacon molecule are taken into account (5). As far as the spatial relationship is concerned, rigid molecules with an optimal fit to one type of receptors will have a higher chance of selectivity in action than flexible molecules, that can fulfill the sterical requirements of more than one type of receptors. Fig. 1 illustrates how in diphenhydramine a highly flexible molecule with both an anticholinergic and an antihistaminic action, introduction of rigidity by suitable ring substitutions results in selectivity to the anticholinergic or selectivity to the antihistaminic activity. Introduction of a t-butyl group in the ortho position results in a high anticholinergic and a low antihistaminic activity, while introduction of a methyl group in the para position results in a high antihistaminic and a low anticholinergic activity. Here we are really approaching the borderline between the structure-activity relationship and structure-action relationship.
Fig. 1. Increase of specificity in action of diphenhydramine derivatives as a result of a decrease in degree of conformational freedom. (After Harms and Nauta, 6)

One may, for instance, expect that the ratio of the activities of optical isomers will be large for highly potent agents, where an optimal fit of the more active isomer with regard to their sites of action may be assumed, while this ratio will be small for less potent agents. This is examplified by the tertiary butyl derivatives and the para methyl derivatives mentioned in Fig. 1. Both derivatives have a centre of asymmetry. For the tertiary butyl derivative, the ratio for the optical isomers is high for the anticholinergic and low for the antihistaminic activity while for the para methyl derivatives this ratio is high for the antihistaminic activity and low for the anticholinergic activity (Fig. 2). These relationships clearly deal not only with the activity but also and especially with the selectivity in action of bioactive agents.
Selectivity in action (8) can be traced back to a complementarity of electron density distribution in the drug molecules and the receptor site on the macromolecule, which is inherent to the specificity in interaction (9).

The objective of the investigations described in this thesis is to contribute to the insight in the factors determining specificity in drug-receptor interaction. Albumin has been used as a model system as to simulate such interactions. The bovine serum albumin used is a properly defined protein (MW 66,000) (10, 11) available in high purity (11). It displays specific (12, 13) as well as non-specific (14, 15) binding of drugs.

Section I deals with the specificity in hydrophobic interaction as investigated by binding of a series of fatty acids to albumin. In the literature many examples are reported of a correlation between lipophi-
licity of drugs and their activity. The same holds true for the affinity of drugs to proteins. Since lipophilicity, if expressed in terms of partition coefficients (3), is practically independent of the spatial arrangement of the groups in the molecules it seems doubtful whether it can lead to specificity in action. However, the hydrophobic binding areas present on or in the protein may be assumed to have a limited size, taken into account the size of the constituting hydrophobic amino acid side chains. This might well imply that nevertheless a more detailed analysis might show an involvement of hydrophobicity in the specificity of action. The possibility of hydrophobic contributions to selectivity in action has been approached by measuring binding constants of fatty acids. In the case of extensive, quasi continuous, hydrophobic binding areas, a monotonous increase of the binding constant may be expected with each addition of a methylene group to the fatty acid. In the case, however, of more restricted hydrophobic areas such as postulated before, one expects discontinuities in this increase there where the fatty acid chain leaves a hydrophobic area and must bridge a more polar area to reach a possible neighbouring hydrophobic area again.

Section II presents a study of the selectivity in binding of acetazolamide (a renal contrast medium) to different binding sites of albumin on basis of nuclear magnetic resonance (nmr). In the first paper of this section attention is focussed upon the mobility of the methyl group of acetazolamide in relation to the aromatic ring. The anisotropic rotation of the methyl group causes a clear non-exponential relaxation of the magnetization which disappears when acetazolamide binds to albumin. Another feature of the nmr spectrum, dealt with in the second paper of this section, is the dependence of linewidth on exchange rates namely the rate of exchange of drug molecules between bound and unbound state (16). On this basis the selectivity acetazolamide displays for the different binding sites of albumin has been explored in relation to the rate constants involved in the binding.

Whether drug-protein binding interferes with, for example, active renal excretion, greatly depends on the rate constants involved in the binding. This matter is discussed in the third paper of this section.

NMR offers the possibility for estimating the mobilities of the different groups of the pharmacon molecule when bound to the receptor (17). This opens perspectives for estimating the participation of the different groups in the binding process. Such investigations are still in its in-
fancy. Up to now efforts to gain information on group participation in
drug-receptor interaction have been mainly restricted to the study of
the difference in activity of stereoisomers.

Section III deals with an analysis of stereoselectivity as a basis
for the mechanism of specific interactions (5). As elucidated before,
for highly active compounds with a centre of asymmetry, for one isomer
a high degree of complementarity and thus a close fit between that mo­
lecule and its site of action must be assumed. This then is impossible
for the other isomer, such that a large activity ratio is to be expected.
For poorly active compounds with a centre of asymmetry a poor fit may be
assumed also for the more active isomer, such that in that case the ac­
tivity ratio of both isomers will tend to become 1. This should hold true
in general for interactions of bioactive molecules such as drugs and
enzyme substrates with their sites of action. A survey of relevant data
from the literature, presented in the first paper of this section,
clearly supports this concept. In the second paper the concept is ra­
tionalized on basis of a model dealing with drug receptor interaction
on a submolecular level.
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SECTION I
SPECIFICITY IN HYDROPHOBIC INTERACTION
Thin-layer chromatographic separation of free fatty acids

Analysis and purification of radioactively labelled fatty acids

J. F. RODRIGUES DE MIRANDA and T D EIKELBOOM

Department of Pharmacology, University of Nijmegen, Nijmegen (The Netherlands)

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In a research project of this laboratory, binding of fatty acids to bovine serum albumin is used as a model system for studying drug-biomacromolecule interaction. Determination of the concentration of free and bound fatty acids in equilibrium-dialysis experiments can be accomplished by using $^{14}$C-labelled fatty acids, although in the dialysis experiments, the $^{14}$C-labelled fatty acids are diluted many times with the $^{12}$C-isotope analogues, the binding of the labelled compounds is monitored. For this reason, the labelled fatty acids must be essentially free from contaminating radioactive compounds.

To check their purity, chromatography is chosen, as this technique can be applied for analysis as well as purification of the compounds. In order to limit the number of operations with radioactive material before and after the purification, there is a preference for analysis of the free fatty acids (FFA) rather than their derivatives. Moreover, as the relative change in physico-chemical properties (lipophilicity) is larger in a series of FFA than in their derivatives or salts, chromatographic separation of the FFA is preferable. Paper$^1$ and thin-layer$^2$ chromatographic (TLC) methods described for separating FFA as ammonium salts report $R_f$ values for the C$^7$ to C$^9$ acids that are too close to allow easy purification.

The difficulty in obtaining reproducible $R_f$ values in TLC with methyl acetate-2.5% aq. ammonia (95:5, v:v) as developing solvent was another factor that persuaded us to seek an alternative chromatographic method.

The excellent gas chromatographic$^3$ and electrophoretic$^4$ separation of FFA cannot easily be adapted to the preparative separation of radioactive FFA. This contrasts with TLC, which, in addition, is relatively inexpensive and requires only compact equipment$^5$.

As silanised silica gel has become commercially available for TLC, it is now possible to separate FFA by reversed-phase chromatography$^6$ Thus, it appeared to us to be worthwhile to look for a simple solvent system for use with this lipophilic adsorbent. As will be shown, we have succeeded in obtaining good separations for the C$^2$ to C$^9$ FFA with a methanol-water system, and have devised a system satisfactory for the purification of $^{14}$C-labelled C$^5$, C$^6$ and C$^7$ FFA.
MATERIALS

The propionic (C₃), butyric (C₄) and hexanoic (C₆) acids were obtained from Baker (Deventer, The Netherlands) The pentanoic (C₅) acid was from Koch-Light (Colnbrook, Great Britain) and the heptanoic (C₇) acid was from Fluka (Buchs, Switzerland) The purity of these acids was checked by gas chromatography at 200° on a column of Porapak Q and was found to be better than 99% by the internal-standard procedure

The [1-¹⁴C]-labelled acids, as sodium salts, were obtained from The Radiochemical Centre (Amersham Great Britain) and had specific activities ranging from 10-20 mCi/mmol These labelled salts were stored at —20° as stock solutions in methanol containing 50 μCi/ml

The TLC plates (20 × 20 cm) were prepared with Desaga equipment (Desaga, Heidelberg, G F R) according to the specifications for the silica gel (HF 254, silanised, E Merck, Darmstadt, G F R)

The scintillation liquid, Insta-gel, was from Packard (Brussels, Belgium) and counting was performed on a Packard Tricarb liquid scintillation spectrometer The direct-contact X-ray film was Kodak RP 54

All other materials used were from E Merck

METHODS AND RESULTS

The liquid non-labelled FFA were diluted five times with methanol before being applied to the plate For the analytical separation, 10 μl of the stock solution of the radioactive acid (C₉) sodium salt) was placed in the tip of a conical tube, and 1 μl of undiluted FFA mixture (C₉, C₁₀, C₁₁ — 1 1 1) was added This mixed sample was sufficient for about five spots of diameter 3 mm All processes involving FFA were carried out at 4°C

For the preparative separation of radioactive FFA, 250 μl of the radioactive stock solution was placed in the tip of a conical tube, and the solvent was evaporated at 60° by aspirating off the vapor The tip was then rinsed with 3 μl of 1 N hydrochloric acid in methanol, and the solution was applied as a “zone” of 4 cm in length The initial spots were placed about 2 cm from the edge of the plate, and the solvent front was allowed to move 10 cm above the spots All solutions were applied to the plate at 4°, and development was carried out at the same temperature

Detection of the non-radioactive FFA was by spraying with a saturated solution of methyl red in 0.2% methanolic sodium hydroxide Detection of the radioactive FFA was by autoradiography or by zone analysis With autoradiographic detection, the plates were first sprayed with the indicator solution, carefully dried and then sprayed with a solution of a fluid adhesive (Lero) in chloroform (this increased the resistance of the adsorbent to mechanical damage) Next, an X-ray film was placed in direct contact with the adsorbent, the contact time being 24 h for 0.05 μCi per spot The zone analysis was performed by scraping the adsorbent from the wet plate in 3-mm zones at 4° (see ref 9) With an analytical separation, the adsorbent of each zone was transferred to a counting phial containing 4 ml of water and 10 ml of Insta-gel, and the radioactivity in each phial was counted For preparative separation, the
adsorbent from each zone was transferred to a conical centrifuge tube containing 1 ml of methanol, and the mixture was stirred.

To determine the position of the main peak, 1-μl aliquots of the supernatant solution in each tube were transferred to a counting phial containing 10 ml of Instagel, and the radioactivity in each phial was counted. The fractions containing the peak concentration of the acid were centrifuged at 3000 g, and the supernatant solution containing the purified acid was removed with a pasteur pipette. To check that the lower FFA had not volatilised during the processes preceding autoradiography, recovery experiments with [1-14C]propionic acid were carried out, recovery of radioactivity was 97 ± 2%.

Development of a suitable solvent system

The solvent system dioxan–water–formic acid (60:35:5), although having a high resolving power for the higher FFA, is less suitable for separation of the lower FFA, this is mainly due to interference by this solvent system with the detection method used.

This disadvantage does not occur with the solvent system methanol–water, which gives excellent separation of the C3 to C9 FFA on silanised silica gel (see Fig 1a). For optimal resolution, the methanol–water ratio depends on the length of the carbon chain of the acid being separated. The dependence of the $R_f$ value on the methanol–water ratio is shown in Fig 2 for the eight FFA investigated, from the curves in Fig. 2, an optimal ratio can be selected for a given mixture of FFA.

![Chromatogram of FFA on silanised silica gel](image)

**Fig 1 Chromatogram of FFA on silanised silica gel.** Development time, 1 h at 4°C, spray reagent, saturated methyl red solution in 0.2% methanolic sodium hydroxide (a) Non-radioactive FFA, solvent methanol–water (35:65). 1 – propionic acid, 2 – butyric acid, 3 – pentanoic acid, 4 – hexanoic acid, 5 – heptanoic acid, 6 – octanoic acid, 7 – mixture of propionic and butyric acids, 8 – mixture of butyric, pentanoic and hexanoic acids, 9 – mixture of hexanoic, heptanoic and octanoic acids. (b) Autoradiogram of 14C-labelled fatty acids, solvent methanol–water (40:60). 1 – propionic acid, 2 – mixture of butyric, pentanoic and hexanoic acids to which pure [14C]butyric acid is added, 3 – mixture of butyric, pentanoic and hexanoic acids to which pure [14C]pentanoic acid is added, 4 – heptanoic acid. The hatching indicates the area in which a red colour is visible after spraying. The blackness corresponds to that on the original autoradiogram and indicates the position of radioactive material. For further details, see text.
Fig 2 Dependence of the $R_f$ values of FFA on the methanol-water ratio. The curves permit selection of an optimal resolving ratio for a given mixture of FFA.

Fig 3 Autoradiograms of $^{14}$C-labelled FFA. (a) 1, 3 — mixtures of propionic, butyric and pentanoic acids, 2 — the same, but with the labelled butyric acid being analyzed added to the mixture. Solvent: methanol-water (20:80). (b) 4, 6 — mixtures of pentanoic, hexanoic and heptanoic acids, 5 — the same, but with the labelled hexanoic acid being analyzed added to the mixture. Solvent: methanol-water (50:50). (c) 7, 9 — mixtures of hexanoic, heptanoic and octanoic acids, 8 — the same, but with the purified $^{14}$C-heptanoic acid added to the mixture. For further details, see legend to Fig 1.
Analysis of labelled free fatty acids

The radioactive FFA were applied to the plate in the way described for the analytical separation: spots of non-radioactive mixtures were applied on either side of the radioactive spots to serve as reference spots. The detection of the labelled FFA was by autoradiography (see Fig. 1b), and depending on the results, one of the following procedures is carried out.

(a) If the autoradiogram indicates an impurity content much less than 1%, as shown in Fig. 3a, there is no need for further chromatography. The result is confirmed by dilution analysis as the p-bromophenacyl ester.

(b) If the autoradiogram indicates an impurity content of about 1% (as shown in Fig. 3b for hexanoic acid), the FFA has now to be analyzed quantitatively by zone

![Diagram](image_url)

Fig. 4 Zone analysis and preparative separation of 14C-labelled FFA on silanised silica gel. The ordinate gives the percentage of radioactivity recovered per mm of scraped zone, the abscissa gives the Rf value of the zone. (a) Zone analysis of [14C]hexanoic acid, solvent. methanol-water (50:50). The calculated purity is 99.2%. (b) Preparative separation of [14C]heptanoic acid, solvent. methanol-water (50:50). Fractions 11 and 12, containing most of the pure heptanoic acid, were stored for further use.
elution A fresh chromatogram is made by applying the FFA to the plate in the way described for the analytical separation, and detection is by the analytical zone-analysis method (see Fig 4a) The zone analysis indicates, for hexanoic acid, a total amount of contaminating radioactivity of 1 \( \pm 0.2 \) \% o, which is within limits considered as acceptable.

(c) If the autoradiogram indicates an impurity content greater than 1 \% (as for heptanoic acid in Fig 3c), then, if the impurity has been clearly diagnosed, this FFA is directly purified according to the procedure described below.

*Purification of labelled FFA*

The radioactive FFA is applied to the plate as described for preparative separation, and detection is carried out as for preparative zone analysis, this is demonstrated for heptanoic acid in Fig 4b. The fractions 11 and 12, containing pure heptanoic acid, are stored at \(-20\) \(^\circ\C\), the purity of these fractions is confirmed by autoradiography (see Fig 3d).

**DISCUSSION**

The methanol–water solvent system in proportions ranging from 10:90 for \(C_2\) to \(C_4\) FFA up to 60:40 for \(C_7\) to \(C_9\) FFA gives excellent and reproducible resolution of the individual FFA on silanised silica gel. The “comets” of the FFA spots, which can impede interpretation of the purity of the labelled FFA (see Fig 1b), can be avoided by applying mixed spots. When mixed spots are applied, the labelled \(C_n\) FFA is forced to distribute itself between the \(C_{n-1}\), \(C_n\) and \(C_{n+1}\) FFA spots, this reduces “comet” formation dramatically (compare Figs 1b and 3a).

The analytical TLC procedure described has been applied successfully to \(^{14}\)C-labelled \(C_3\) to \(C_7\) FFA. It is noteworthy that, in general, the \(C_n\) FFA appears not to be contaminated with \(C_{n+1}\) or \(C_{n-1}\) FFA, but with some \(C_{n+2}\) and \(C_{n-2}\) FFA.

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THE EXTENT OF HYDROPHOBIC BINDING AREA
studied by fatty acid binding to albumin

University of Nijmegen, Nijmegen, The Netherlands

*Department of Pharmacology
†Department of Biophysical Chemistry
INTRODUCTION

Studies on structure-activity relationships show that hydrophobicity not only plays an important role in the drug distribution, but also in the interaction of the drug with its site of action, the receptor (1). The hydrophobic interaction may contribute substantially to the stability of the drug-receptor complex and the high $pA_2$-values, ranging up to 10 log units as observed, for instance, for anticholinergics and antihistaminics, seem to be a consequence of an effective hydrophobic interaction (2, 3). The effectiveness of such an interaction has probably an upper limit of about 800-1000 cal per CH$_2$ group in the case of an alkyl chain. This appears from studies of the free energy change for the transfer of amphiphilic compounds like alcohols and fatty acids from an aqueous to a lipophilic phase (4).

From the positive correlation between the lipophilicity of a drug and its biological activity, often reported (5, 6), one can, however, not conclude, that the hydrophobic binding area on the receptor makes no spatial demands to the apolar part of the drug molecule. Different studies have been devoted to a systematic exploration of the steric and spatial structure of the hydrophobic binding regions on proteins (7, 8, 9, 10) and in general, lengthening of the aliphatic side chain of a drug molecule does not imply simply an increase in affinity (8, 9). In those cases the essential part of the receptor is built up by proteins this finding is not unexpected given the fact of the relatively short side chains of the lipophilic amino acids and the small chance that three or more of these side chains can form hydrophobic binding areas at the surface of the protein in aqueous solution.

In the studies of Belleau et al. (8) on the binding of alkyl trimethylammonium ions to acetylcholinesterase and in those of Marlow et al. (9) on the binding of alkyl trimethylammonium ions to "antibody binding acetylcholine", the effectiveness of the hydrophobic interaction, expressed as the free energy change per methylene group, seems to be rather low. It has been our aim to explore the hydrophobic binding area in the case of a more effective hydrophobic interaction. As will be shown, the first specific binding site on albumin for fatty acids meets this requirement. Therefore the extent of the hydrophobic binding area on albumin has been measured in terms of alkyl chain.
length by determining the relation between the free energy change of binding and the chain length.

MATERIALS AND METHODS

The propionic (C₃), butyric (C₄), caproic (C₆) and caprylic (C₈) acids were obtained from Baker, Deventer, the Netherlands, the valeric (C₅) acid from Koch-Light Laboratories, Colnbrook, Bucks, England, and the heptanoic (C₇) acid from Fluka AG, Buchs, S.G., Switzerland. The purity of the fatty acids was checked gaschromatographically on a porapaque-Q-column with an oven temperature of 200°C and found to be over 99% using the internal standard procedure.

The radioactive compounds were all from the Radiochemical Centre, Amersham, England. The labelled fatty acids-1-C¹⁴-sodium salts have a specific activity ranging from 10-20 mCi/m mole. They were stored at -20°C as stock solutions containing 10 μCi/ml and an excess of NaOH. Na²⁴ was supplied as saline containing 3 μCi/2.5 ml. The radiochemical and chemical purity of the fatty acids-1-C¹⁴ were checked by thin-layer chromatography (11). Crystalline bovine serum albumin (BSA) was purchased from Povite, Amsterdam, the Netherlands (batch 462, 517 and 625) and from Nutritional Biochemicals Corporation, NBC, Cleveland, Ohio, USA (batch 3404). The concentration of the albumin solution was measured spectrophotometrically at 279 nm on a Zeiss PMQ-II (E¹₇₉ = 6.67). The pH of the solutions was measured on a Radiometer 26 pH-meter. The scintillation liquid, Instagel, was from Packard, Brussels, Belgium. The samples were counted with standard deviations of 0.2% or less in a Packard Tricarb liquid scintillation spectrometer, model 3380. The cellulose acetate membranes were from AKU, Arnhem, the Netherlands. Before use they were thoroughly rinsed in demineralized water. Na²⁴ was counted on a Philips γ-scintillation counter PW 4003. All other materials were from E. Merck AG, Darmstadt, Germany.

The binding of the fatty acids to the protein was determined in
equilibrium dialysis experiments. The dialysis apparatus was made after a design of A.S.V. Burgen (12). It consists of six pairs of shallow teflon cells of 3.5 ml volume each separated by a cellulose acetate membrane and clamped together (Fig. 1).

The albumin solution is brought at one side of the membrane, the fatty acid solution, containing tracer amounts of 1-C\textsuperscript{14}-labelled fatty acids, at the other side. If not otherwise stated, all solutions were made in a phosphate buffer pH 6.9, ionic strength 0.1, containing 0.02% NaN\textsubscript{3} as a bacteriostatic. The apparatus rotates at the rate of 1 rpm in a thermostatic water-bath. Depending on the chain length of the fatty acid, equilibrium was reached within 6-8 hours at 30\textdegree C and within 16-20 hours at 4\textdegree C.

**DETERMINATION OF FATTY ACID CONCENTRATION AT EQUILIBRIUM**

After equilibrium was reached 500 µl aliquots from the solutions on
both sides of the membrane were taken. To each aliquot 11 ml of an Instagel-water mixture (10 : 1) were added, and the radioactivity was counted and compared with a reference with a known fatty acid content. The counting efficiency is 88.3% (SD 0.1) and independent of the BSA and fatty acid concentrations in the range applied. By applying the appropriate correction for the Donnan-ratio (13, 14) and the plasma water (15), the concentration of F.A. bound to albumin can be calculated by subtracting the free concentration (one side of the membrane) from the total concentration (other side of the membrane).

DETERMINATION OF THE PROTEIN CONCENTRATION

Especially at higher albumin concentrations, i.e. 6.6%, an osmotic dilution of the albumin solution occurs during dialysis. For this reason albumin concentrations were determined after equilibrium has been reached. The Donnan ratio's occurring at the different circumstances were determined by the distribution of Na$^{24}$ across the membrane.

THEORETICAL BACKGROUND

MULTIPLE BINDING

The association between a small molecule S and a binding site P on a protein can be described by the following reaction equation:

$$P + S \rightleftharpoons PS \quad K = \frac{[PS]}{[P][S]} \quad M^{-1}$$

(1)

If the protein contains n independent and identical sites it holds
true that:

\[
\begin{align*}
[PS] &= \bar{r} P_0 \\
\text{and} \\
[P] &= (n - \bar{r}) P_0
\end{align*}
\]

in which \( \bar{r} \) is the average number of substrate molecules bound to the protein molecule and \( P_0 \) is the total protein concentration. Substitution of eqs. 2 and 3 in eq. 1 results in:

\[
\frac{\bar{r}}{n - \bar{r}} = K[S] \tag{4}
\]

or

\[
\frac{\bar{r}}{[S]} = Kr - Kn \tag{5}
\]

A plot of \( \bar{r}/[S] \) versus \( \bar{r} \) (Scatchard plot) yields a straight line with intercepts \( nK \) and \( n \). From equation 4 it follows that:

\[
\frac{\bar{r}}{n} = \frac{K[S]}{1 + K[S]} \quad \text{or} \quad \bar{r} = \frac{nK[S]}{1 + K[S]} \tag{6}
\]

or taking the reciprocal:

\[
\frac{1}{\bar{r}} = \frac{1}{n} + \frac{1}{nK} \cdot \frac{1}{[S]} \tag{7}
\]

A plot of \( 1/\bar{r} \) versus \( 1/[S] \) (Lineweaver-Burk plot (16)) yields a straight line with intercepts \( 1/n \) and \( -1/K \).

If there is more than one class of binding sites on the surface of the protein such that class 1 has \( n_1 \) sites and an intrinsic association constant \( K_1 \), class 2 has \( n_2 \) sites and an intrinsic association constant \( K_2 \), etc., then eq. 6 may be generalized as follows (17, 18):
with \( \bar{r}_i \) being the average number of substrate molecules bound to class i. In fact, \( K \) is an apparent intrinsic association constant in which activity coefficients and electrostatic interaction terms are combined. A plot of \( \frac{\bar{r}_T}{[S]} \) versus \( \bar{r}_T \) will now give a curved line.

**ANALYSIS OF BINDING CURVES**

If the experimental points do not yield a straight line, then the curve can be interpreted as being composed of two or more straight lines corresponding to different classes of binding sites. Rosenthal has described a graphical procedure to analyze such curves and to estimate the corresponding \( n_i^2 \) and \( K \)-values (19). We used these as starting values in our computerized curve-fitting procedure based on a gradient method for the non-linear parameter \( K \) and a linear regression method for the linear parameter \( n \) (20). In general, the choice of the number of classes of binding sites (binding model) with which the experimental results can be described is somewhat arbitrary. True, the presence of more than one class of binding sites can, provided factors such as electrostatic, are constant or may be neglected, unambiguously be deduced from the deviation of the Scatchard plot from a straight line. However, the errors in the experimental data rarely allow a differentiation between a binding model with three, four or more classes of binding sites. In the analysis of our experimental results the criterion for extending the number of classes was a statistical one, i.e. the number of classes were extended as long as a significant decrease in the residual sum of squares, \( \chi^2 \), occurred. The different models used are characterized and indicated by four digits, e.g. 312.2. The number of non-linear parameters, \( K_i \), estimated by iteration is indicated by the first digit (i.e., 3 in the example). The number of those linear parameters, \( n_i \), which were estimated by iteration is indicated by the second digit (i.e., 1 in the example).
The number of linear parameters with a chosen fixed integral value is indicated by the fourth digit (i.e., 2 in the example). The third digit gives the number of iteration terms; the terms in equation 8 in which the $n_i$ values are fixed are considered as one iteration term. If the value of the first digit is lower than the third, this indicates the assumption of a partition term (or class with $K_i S << 1$) in excess to the classes of binding sites.

RESULTS

BINDING CONDITIONS

Typical results obtained from the binding experiments are shown in Fig. 2 as Scatchard plots for the binding of valerate to Povite albumin at 4° and 30°C. As appears from these curves, at least two classes of binding sites are involved in the binding.

To investigate the influence of ionic strength on the binding, experiments were carried out with different salt conditions. This is demonstrated in Fig. 3 for the binding of valerate to albumin. The observed influence of the different anions on the binding of the valerate anion is compatible with the different affinities of the electrolyte anions for albumin (21). Since phosphate anions appear to interfere least with the binding of organic anions (21) it was used in all subsequent experiments.

Since drug-receptor interaction is assumed to be specific, special attention was given to the binding of the fatty acid to the first, specific, binding site. Therefore it was essential to check whether the albumin used in the experiments was free from native fatty acids (22-24). To this end the binding of hexanoic acid to BSA from different manufacturers was measured before and after charcoal defatting (25). In Fig. 4 the results obtained with NBC albumin are shown as
Fig. 2. Typical results obtained from dialysis binding experiments:
Scatchard plots of the binding of valerate to untreated Povite albumin at 4°C (●) and at 30°C (○). (Solution conditions: 0.05 M phosphate buffer pH 6.9) The solid lines represent least squares fits to the experimental points based on equation 8, model 223.1 (see text).
Fig. 3. Scatchard plots indicating the influence of type and concentration of electrolytes on the binding of valerate to untreated NBC albumin at 30°C, pH 6.9. 0.1 M KCl (•); 0.25 M KCl (□); 0.05 M phosphate buffer (×).
Fig. 4. Scatchard plots indicating the influence of charcoal defatting on the binding of hexanoate to albumin at 4°C. The lines represent least squares fits to the experimental values, based on equation 8 model 222.0. The solid line is obtained with untreated NBC albumin; $K_1 = 2.5 \pm 0.3 \times 10^4 \text{ M}^{-1}$, $n_1 = 1.4 + 0.1$. The dashed line is obtained with untreated Povite albumin; $K_1 = 3.8 \pm 0.3 \times 10^4 \text{ M}^{-1}$, $n_1 = 1.9 + 0.1$. The points indicate the results of one single experiment; (□) untreated NBC albumin; (■) charcoal defatted NBC albumin. (Solution conditions as in Fig. 2).
Fig. 5. Scatchard plots indicating the influence of charcoal defatting on the binding of hexanoate to Povite albumin at 4°C. The solid lines represent least squares fits to the experimental points based on equation 8, model 222.0. (●) Untreated Povite albumin, batches 462 and 517; $K_1 = 3.8 (± 0.3) \times 10^4 \text{ M}^{-1}$, $n_1 = 1.9 (± 0.1)$, (x) charcoal-defatted Povite albumin, batch 517; $K_1 = 3.2 (± 0.2) \times 10^4 \text{ M}^{-1}$, $n_1 = 2.0 (± 0.1)$. (Solution conditions as in Fig. 2).

hexanoate to Povite albumin. From these curves it is evident that untreated Povite albumin binds stronger than untreated NBC albumin (Povite: $K_1 = 3.8 \times 10^4 \text{ M}^{-1}$, $n_1 = 1.9$; NBC: $K_1 = 2.5 \times 10^4 \text{ M}^{-1}$, $n_1 = 2.0$).
η = 1.4). The points, indicated in the figure, represent the results of one single dialysis experiment in which under identical circumstances, the binding of hexanoate to untreated NBC albumin is compared with the binding of charcoal-defatted NBC albumin. Clearly, the binding characteristics of charcoal-defatted NBC albumin become identical to those of untreated NBC albumin.

Fig. 5 shows the binding of hexanoate to untreated Povite albumin compared with the binding to charcoal-defatted Povite albumin. Evidently, the binding characteristics of Povite albumin are affected by charcoal defatting only to a minor extent. From these results and those obtained with other fatty acids and some renal contrast media, it was concluded that Povite albumin, contrary to NBC albumin, does not contain interfering lipid impurities. For this reason, if not otherwise stated, untreated Povite albumin was used in our experiments.

ANALYSIS OF THE BINDING DATA

Curves were fitted to the experimental points (e.g. solid lines in Fig. 2) using equation 8 to describe the binding. The values for the parameters, \( n_i \) and \( K_i \), obtained from this curve fitting procedure depend on the number of classes of binding sites chosen to describe the binding. Table 1 summarizes, for the case of valerate, the different \( n \)- and \( K \)-values obtained from the different models used to describe the binding. Each model, indicated on a separate line in Table 1, is characterized by the four digits in the first column as was indicated above. In the next columns the values and standard errors for \( n_i \) and \( K_i \) of the \( i \)th class are given. The third column from the end gives the degrees of freedom, \( v \), in the curve-fitting procedure. The residual sum of squares, \( \chi^2 \), in the next column, is used as a measure for the goodness of fit. It can be compared with tabulated levels of significance, i.e. \( \chi^2 \text{5%} \).

As appears from this table, the calculated \( K \)-values clearly depend on the model chosen. As far as the first class of binding sites is concerned, the product of \( n_1 \) and \( K_1 \) is, however, remarkably constant. So if the number of binding sites in the first class is fixed (e.g. on 1) a \( K_1 \)-value is obtained which is practically independent of the
**Table 1**

<table>
<thead>
<tr>
<th>Model</th>
<th>$n_1$</th>
<th>$K_1$ ($10^4$ M⁻¹)</th>
<th>$n_2$</th>
<th>$K_2$ ($10^4$ M⁻¹)</th>
<th>$n_3$</th>
<th>$K_3$ ($10^4$ M⁻¹)</th>
<th>$n_4$</th>
<th>$K_4$ ($10^4$ M⁻¹)</th>
<th>$\nu$</th>
<th>$\chi^2$</th>
<th>$\chi^2_{5%}$</th>
</tr>
</thead>
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<tr>
<td>222 0</td>
<td>1.36 (0.05)</td>
<td>3.3 (0.2)</td>
<td>5.2 (0.4)</td>
<td>0.014 (0.002)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>54</td>
<td>136</td>
<td>72</td>
</tr>
<tr>
<td>233 0</td>
<td>1.13 (0.05)</td>
<td>4.1 (0.2)</td>
<td>2.2 (0.2)</td>
<td>0.08 (0.02)</td>
<td>0.0078 (0.0007)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>53</td>
<td>97</td>
</tr>
<tr>
<td>333 0</td>
<td>1.04 (0.07)</td>
<td>4.5 (0.4)</td>
<td>1.6 (0.2)</td>
<td>0.15 (0.05)</td>
<td>9 (3)</td>
<td>0.0018 (0.0009)</td>
<td></td>
<td></td>
<td>52</td>
<td>66</td>
<td>70</td>
</tr>
<tr>
<td>344 0</td>
<td>0.9 (0.2)</td>
<td>5.2 (0.9)</td>
<td>0.9 (0.3)</td>
<td>0.5 (0.5)</td>
<td>2.3 (0.5)</td>
<td>0.02 (0.02)</td>
<td>0.006 (0.001)</td>
<td></td>
<td></td>
<td>51</td>
<td>60</td>
</tr>
<tr>
<td>444 0</td>
<td>0.9 (0.2)</td>
<td>4.9 (0.1)</td>
<td>1.0 (0.2)</td>
<td>0.4 (0.3)</td>
<td>4 (2)</td>
<td>0.01 (0.01)</td>
<td>0.6 (2.8)</td>
<td>0.002 (0.001)</td>
<td></td>
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<td>59</td>
</tr>
<tr>
<td>223 1</td>
<td>1</td>
<td>4.7 (0.1)</td>
<td>2.1 (0.1)</td>
<td>0.12 (0.01)</td>
<td>0.0085 (0.0006)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>54</td>
<td>75</td>
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<tr>
<td>323 1</td>
<td>1</td>
<td>4.7 (0.1)</td>
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<td>0.18 (0.03)</td>
<td>9 (3)</td>
<td>0.0021 (0.0009)</td>
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<td></td>
<td>53</td>
<td>66</td>
<td>71</td>
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<tr>
<td>334 1</td>
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<td>4.6 (0.1)</td>
<td>1.0 (0.4)</td>
<td>0.3 (0.2)</td>
<td>2.2 (0.6)</td>
<td>0.02 (0.02)</td>
<td>0.006 (0.001)</td>
<td></td>
<td></td>
<td>52</td>
<td>61</td>
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<td>1</td>
<td>4.6 (0.1)</td>
<td>1</td>
<td>0.32 (0.03)</td>
<td>6.3 (0.6)</td>
<td>0.0052 (0.0008)</td>
<td></td>
<td></td>
<td>54</td>
<td>74</td>
<td>72</td>
</tr>
<tr>
<td>322 2</td>
<td>1</td>
<td>4.6 (0.1)</td>
<td>1</td>
<td>0.28 (0.03)</td>
<td>2.2 (0.6)</td>
<td>0.018 (0.007)</td>
<td>0.006 (0.001)</td>
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<td>61</td>
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<td>0.29 (0.03)</td>
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<td>0.010 (0.005)</td>
<td>0.6 (2.5)</td>
<td>0.002 (0.001)</td>
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<td>52</td>
<td>60</td>
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</tbody>
</table>

Different $n$- and $k$-values obtained from the different models used to describe the binding of valerate to bovine albumin. Each model, indicated on a separate line in Table 1, is characterised by the four digits in the first column (e.g., 312.2). The number of non-linear parameters, $K_i$, estimated by iteration is indicated by the first digit (i.e., 3 in the example). The number of those linear parameters, $n_i$, which were estimated by iteration is indicated by the second digit (i.e., 1 in the example). The number of linear parameters with a chosen fixed integral value is indicated by the fourth digit (i.e., 2 in the example). The third digit gives the number of iteration terms; the terms in equation 8 in which the $n_i$ values are fixed, are considered at one iteration term. If the value of the first digit is lower than the third, this indicates the assumption of a partition term (or class with $K_iS << 1$) in excess to the classes of binding sites. In the next columns the values and standard errors for $n_i$ and $K_i$ of the $i$th class are given. The third column from the end gives the degrees of freedom, $\nu$, in the curve fitting procedure. The residual sum of squares, $\chi^2$, in the next column, is used as a measure for the goodness of fit. It can be compared with tabulated levels of significance, i.e. $\chi^2_{5\%}$. 
<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>n_1</th>
<th>K_1 (10^4 M^-1)</th>
<th>n_2</th>
<th>K_2 (10^4 M^-1)</th>
<th>n_3</th>
<th>u</th>
<th>\chi^2</th>
<th>\chi^2 5%</th>
</tr>
</thead>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na-Propionate</td>
<td>1</td>
<td>0.009 (0.004)</td>
<td>0.9 (0.2)</td>
<td>0.027 (0.009)</td>
<td>0.004 (0.001)</td>
<td>14</td>
<td>1</td>
<td>24</td>
</tr>
<tr>
<td>Na-Butyrate</td>
<td>1</td>
<td>1.80 (0.02)</td>
<td>1.5 (0.1)</td>
<td>0.064 (0.008)</td>
<td>0.0038 (0.0006)</td>
<td>38</td>
<td>13</td>
<td>53</td>
</tr>
<tr>
<td>Na-Valerate</td>
<td>1</td>
<td>4.7 (0.1)</td>
<td>2.1 (0.1)</td>
<td>0.12 (0.01)</td>
<td>0.0085 (0.0006)</td>
<td>54</td>
<td>75</td>
<td>72</td>
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<tr>
<td>Na-Hexanoate</td>
<td>1</td>
<td>6.8 (0.3)</td>
<td>2.2 (0.2)</td>
<td>0.30 (0.05)</td>
<td>0.018 (0.002)</td>
<td>41</td>
<td>105</td>
<td>57</td>
</tr>
<tr>
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<td>28 (1)</td>
<td>3.3 (0.1)</td>
<td>0.45 (0.08)</td>
<td>0.038 (0.004)</td>
<td>38</td>
<td>118</td>
<td>53</td>
</tr>
<tr>
<td>Na-Octanoate</td>
<td>1</td>
<td>164 (9)</td>
<td>3.4 (0.4)</td>
<td>1.0 (0.2)</td>
<td>0.12 (0.02)</td>
<td>20</td>
<td>37</td>
<td>32</td>
</tr>
<tr>
<td>Temperature 30°C</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na-Propionate</td>
<td>1</td>
<td>0.063 (0.005)</td>
<td>1 (1)</td>
<td>0.01 (0.01)</td>
<td>0.003 (0.004)</td>
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<td>24</td>
</tr>
<tr>
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<td>0.83 (0.02)</td>
<td>1.3 (0.2)</td>
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<td>0.006 (0.001)</td>
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<td>50</td>
</tr>
<tr>
<td>Na-Valerate</td>
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<td>0.0093 (0.0007)</td>
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<td>48</td>
<td>50</td>
</tr>
<tr>
<td>Na-Hexanoate</td>
<td>1</td>
<td>3.2 (0.1)</td>
<td>2.7 (0.2)</td>
<td>0.19 (0.03)</td>
<td>0.017 (0.002)</td>
<td>48</td>
<td>98</td>
<td>65</td>
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<tr>
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<td>4.6 (0.3)</td>
<td>0.22 (0.03)</td>
<td>0.032 (0.003)</td>
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<td>47</td>
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<tr>
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<td>1</td>
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<td>5.0 (0.3)</td>
<td>0.50 (0.04)</td>
<td>0.10 (0.01)</td>
<td>20</td>
<td>8</td>
<td>32</td>
</tr>
</tbody>
</table>

For explanation of the symbols see text table 1.
### Binding of fatty acids to BSA

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>$n_1$</th>
<th>$K_1$ ((10^4 \text{ M}^{-1}))</th>
<th>$n_2$</th>
<th>$K_2$ ((10^4 \text{ M}^{-1}))</th>
<th>$n_3$</th>
<th>$K_3$ ((10^4 \text{ M}^{-1}))</th>
<th>$\nu$</th>
<th>$\chi^2$</th>
<th>$\chi^2 5%$</th>
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</thead>
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<tr>
<td>Na–Propionate</td>
<td>1</td>
<td>0.104 (0.002)</td>
<td>1.7 (0.1)$^a$</td>
<td>0.014 (0.001)</td>
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<tr>
<td>Na–Butyrate</td>
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<td>1.78 (0.02)</td>
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<td>0.094 (0.009)</td>
<td>2.3 (0.3)</td>
<td>0.006 (0.002)</td>
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<td>14</td>
<td>53</td>
</tr>
<tr>
<td>Na–Valerate</td>
<td>1</td>
<td>4.6 (0.1)</td>
<td>1</td>
<td>0.32 (0.03)</td>
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<td>0.0052 (0.0008)</td>
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<td>74</td>
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<td>6.0 (0.3)</td>
<td>1</td>
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<td>6.2 (0.6)</td>
<td>0.014 (0.002)</td>
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<td>56</td>
<td>57</td>
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<td>Na–Butyrate</td>
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<td>Na–Valerate</td>
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<td>50</td>
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<td>11 (4)</td>
<td>0.02 (0.02)</td>
<td>19</td>
<td>8</td>
<td>30</td>
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</tbody>
</table>

*For explanation of the symbols see text table 1.

*a* calculated with model 212.1; *b* calculated with model 323.1.
model adopted; moreover, this $K_1$-value, representing the binding of the first fatty acid molecule to albumin, equals the so-called stepwise equilibrium constant defined by Fletcher (26) and can thus be considered independent of the binding of further fatty acid molecules. The experimental data obtained for the five other fatty acids were analyzed in a similar way as for valerate. The results obtained at $4^\circ$ and $30^\circ$C for the two most simple models which in general give reliable $\chi^2$-values are summarized in Tables 2 and 3. Table 2 gives the results obtained with the model 223.1, i.e. two classes of binding sites with $n_1 = 1$ and a partition term. Table 3 gives the results obtained with the model 312.2, i.e. three classes of binding sites with $n_1 = 1$ and $n_2 = 1$.

**DISCUSSION**

Aqueous solution of hydrophobic substances show an apparently anomalous thermodynamic behaviour (27). The enthalpy of solution is contrary to what would be expected negative. This negative enthalpy change, however, is more than counterbalanced by a very large negative entropy of solution; this results in a positive standard free energy change and thus in a small solubility. This anomalous behaviour has been attributed to an effect of the solute on the structure of liquid water (28).

The binding of hydrophobic substances, and probably also of amphiphilic substances like fatty acids, to proteins can be considered as a partial reversal of the dissolution process (4, 28). The standard free energy of transfer, $\Delta G^0$, (4) of the ligand to a protein-binding site can then be written as:

$$\Delta G^0 = -RT \ln K = \Delta H^0 - T\Delta S^0$$ (9)

where $K$ is the equilibrium constant for the binding site and $\Delta H^0$ and
ΔS^0 are the standard enthalpy and entropy changes accompanying the binding process. If the hydrophobic effect dominates in the binding process, it is to be expected that ΔH^0 and ΔS^0 will both be positive.

From Tables 2 and 3 the affinity constants of the fatty acids for the first binding site on albumin, K_1, can be read. The dependence of the thermodynamic parameters, derived from this affinity constants at 4°C and 30°C, on the alkyl chain length of the fatty acids provides information on the specific binding sites.

![Thermodynamic parameters graph](image)

**Fig. 6.** ΔG^0 in kcaL for the binding of fatty acids, H(CH_2)_nCOO^- to Povite albumin plotted as a function of the chain length, n, at 4°C. (Solution conditions as in Fig. 2). Note the occurrence of the plateau at n = 4-5.

In Fig. 6 ΔG^0 at 4°C is plotted as a function of chain length; the black circles represent the values of ΔG^0 calculated from K_1; the standard errors fall within the diameter of the circles. Evidently ΔG^0 does not increase linearly with increasing chain length but a plateau is reached at n = 4 (valerate), and only at n = 6 ΔG^0 further increases. The increment of ΔG^0 per CH_2-group amounts to 1600 cal in the steepest part of the curve and 200 cal in the plateau. For the six fatty acids studied the mean increment per methylene group amounts to 820 cal.
Within experimental error, this value equals that of 825 cal, obtained by Tanford for the free energy change for transfer of undissociated fatty acids from a dilute aqueous buffer solution to liquid n-heptane at 23°C (4). This indicates that the hydrophobic interaction of the alkyl chain of the fatty acid is quite optimal.

As can be seen in Fig. 7, the occurrence of the plateau in the $\Delta G^o$-value for the first binding site is independent of the binding model chosen. In this figure also the $\Delta H^o$ and $\Delta S^o$ values have been plotted as a function of n. The $\Delta H^o$-value is negative and, except for the propionate anion, rather constant so that it seems to reflect predominantly the enthalpy change due to the binding of the carboxylic group. The $\Delta S^o$-
value is positive as has to be expected for a hydrophobic interaction (4). Seeing that it increases with chain length it probably reflects predominantly the entropy change due to the binding of the hydrophobic alkyl chain.

The occurrence of the plateau in the plot of $\Delta G^0$ versus $n$ can be interpreted in two ways:

a. The binding site for fatty acids is built up from at least two hydrophobic binding areas. When assuming that the aliphatic chain binds in a stretched conformation, the extent of the first hydrophobic area is such, that it can accommodate a chain length of three or four carbon atoms. Fatty acids with an alkyl chain length exceeding the three to four methylene groups apparently come across a second hydrophobic binding area located at a distance of about two carbon atoms from the first one.

b. Another way to look at the occurrence of the plateau is to suppose that we are dealing with two types of hydrophobic binding sites. The occurrence of the plateau then reflects the transition from binding of the fatty acids to the first type to binding to the second type of binding sites.

To differentiate between these two possibilities competition experiments have been performed. Competition for a common first binding site can most conveniently be traced by plotting $1/r_T$ versus $1/S$ (Lineweaver-Burk-plot). Fig. 8 gives such plots for the competition of butyrate and propionate for the first binding site of valerate. As appears from these plots the intercept on the $1/r_T$-axis remains the same in the presence of the displacing fatty acid, butyrate or propionate. Moreover, values of the affinity constants of the displacing fatty acids derived from the apparent decrease in the affinity constant of valerate, further referred to as displacement constants, equal, within experimental error, the values of the affinity constants obtained before, from dialysis experiments of the fatty acids alone (compare Tables 2 and 3). This indicates that these fatty acids do have their first binding site in common. The competition of hexanoate and heptanoate for the first binding site of octanoate displayed a similar picture, indicating that also these three fatty acids do have their first binding site in common. The competition of butyrate for the first binding site of octanoate is, however, much less effective and the same
Fig. 8. Lineweaver-Burk plots indicating the competition for the first binding site of valerate on Povite albumin. (●) without displacing compound; (△) in the presence of 10.6 x 10⁻⁴ M propionate; (Ψ) in the presence of 2.5 x 10⁻⁴ M butyrate. (Solution conditions as in Fig. 2)

holds true for the competition of octanoate for the first binding site of butyrate. Although some competitive displacement of butyrate by octanoate and of octanoate by butyrate occurs, the displacement constants thus calculated are an order of magnitude smaller than the affinity constants, obtained from dialysis experiments with the fatty acid alone. Evidently, octanoate and butyrate have not their first binding site in common. Probably, a second binding site of octanoate serves as a first binding site of butyrate and vice versa. Since propionate and butyrate do displace valerate with displacing constants about equal to their Kᵢ's and hexanoate and heptanoate do displace octanoate with dis-
placing constants about equal to their $K_i$'s, the picture emerges that, as supposed above in b., we are dealing with two types of hydrophobic binding sites. The first one, for short-chain fatty acids, can accommodate an alkyl chain with a length up to $n = 4$, and the second one, for long-chain fatty acids, can accommodate an alkyl chain as long as a heptyl chain. This second site, however, is less adapted to bind short-chain fatty acids.

CONCLUSIONS

EXTENT OF HYDROPHOBIC BINDING AREAS

From the results obtained in experiments with fatty acids alone and those performed in the presence of displacing fatty acids, one would conclude that the hydrophobic binding area, forming part of the first specific binding site for fatty acids on albumin, provides an almost optimal hydrophobic interaction possibility. For short-chain fatty acids this binding area seems to be limited in extent. This supports the representation given by Tanford (4). When assuming that the aliphatic chain is bound in a stretched conformation, the extent of this hydrophobic area has to be such, that it can accommodate a chain with a length of three to four carbon atoms. As far as the first binding site for long-chain fatty acids is concerned additional experiments will be needed to explore the extent of this hydrophobic binding area. The fact that this binding site is less adapted to bind short-chain fatty acids might be the consequence of an induced fit occurring on binding.

From a comparison of our data with earlier work (Fig. 9), it now becomes obvious, that the data of Teresi and Luck (29) show the same tendency in having a plateau in $\Delta G^\circ$ at $n = 6$ to $n = 4$. The data of Goodman (30) and Spector et al. (31) for the binding of the longer
Fig. 9. Comparison of $\Delta G^0$-values obtained from the present results with those from literature; (□) present results at 4°C; (●) results from Teresi and Luck (29) at 23°C, (○) Goodman (30) at 23°C, (▲) Spector et al. (31) at 37°C. (The solution conditions are different)
Note that the data of Teresi and Luck show the same tendency in having a plateau at $n = 4-5$.

fatty acids to albumin (Fig. 9) leave open the possibility for the occurrence of a second plateau. Differences in the absolute values of $\Delta G^0$ can be traced back to differences in solution conditions and purity of the albumin as has been demonstrated in Figs. 3 and 4. Also various pharmacological data (8, 9, 32) seem to indicate that the hydrophobic binding area cannot accommodate chains longer than 2 to 4 carbon atoms. So it becomes tempting to postulate that lipophilic binding areas in the neighbourhood of polar groups on the protein in general cannot accommodate groups with a length of a carbon chain of more than four atoms.

AFFINITY CONSTANT AND DISPLACEMENT CONSTANT

From the competition experiments it became obvious, that it does make sense to differentiate, even in the case of closely related compounds like fatty acids, between the affinity constant obtained from the
binding of a drug to a certain protein and the displacement constant of the same drug deduced from a particular competition experiment with the same protein. In addition to competitive inhibition and non-competitive or allosteric inhibition a further possibility may be distinguished, viz. in which the drug shares its first binding site with a non-specific second or third binding site of the inhibitor. In that case the inhibitor displaces the drug competitively, although less effectively than would be expected on basis of the affinity constant of the inhibitor for its own first binding site (33). Reversely, conclusions concerning affinity constants deduced from displacement experiments (34, 35) need not to refer to the first specific binding site of the displacing drug.

ACKNOWLEDGEMENT

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REFERENCES


SECTION II
SPECIFICITY STUDIED BY NUCLEAR MAGNETIC RESONANCE
Nonexponential Relaxation of the Methyl Protons of Acetrizoate in Solution

J. F. Rodrigues de Miranda* and C. W. Hilbers†

University of Nijmegen, Nijmegen, The Netherlands

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The time dependence of the magnetization of the methyl group of acetrizoate dissolved in D_2O is found to be strongly nonexponential. This effect is attributed to the influence of cross-correlations and is in nice agreement with the theory of Hubbard and its extended form presented by Werbelow and Marshall. The rotational diffusion of the methyl group around its axis is estimated to be 30 times as fast as the overall rotation of the whole molecule. In the presence of BSA the nonexponential relaxation effect disappears. In addition it is shown that, for a symmetric top molecule with the axis of the methyl group along the molecular symmetry axis and the methyl group rotation in the extreme narrowing limit, analytical expressions can be derived for the time decay of the magnetization, when the rotation of the molecule perpendicular to this axis is not in the limit of extreme narrowing. There is, however, a lower limit to the rate of this motion.

INTRODUCTION

Recently a number of papers have been devoted to the study of the relaxation behavior of methyl groups (1–4). In particular the possible influence of cross-correlation has aroused some attention since it may give rise to a nonexponential relaxation pattern. This effect will only become manifest when the overall motion of the methyl group is highly anisotropic (1, 2, 4) and may therefore be of potential interest to protein and membrane studies. It can, however, be masked by other mechanisms like intermolecular dipole–dipole and spin–rotation relaxation. Nevertheless, since cross-correlation terms will always be present when the spin system consists of three or more rigidly fixed spins (rigidly fixed with respect to each other), it should be quite a general phenomenon. The effect has been observed in solids (5, 6), but it is somewhat surprising that in solutions it has only been measured in a few rare instances (6, 7). There is even some confusion in one case whether indeed the observed nonexponential relaxation should be attributed to cross-correlation effects (4, 6), while in the other case (7) the effect is largely masked by spin–rotation relaxation contributions. This would imply that the cross-correlation terms are apparently small or not very important.

During the course of our investigation of the binding of the renal contrast medium acetrizoate to serum albumin, we found that the methyl group of the acetrizoate exhibited a pronounced nonexponential relaxation behavior. In this molecule the effect of the cross-terms clearly becomes manifest and it is anticipated that acetrizoate is

* Department of Pharmacology.
† Department of Biophysical Chemistry.
EXPERIMENTAL

The acetrizoate (triognost®) used in these experiments was kindly supplied by Draga (Draga, Diemen, The Netherlands). Bovine serum albumin (BSA) was purchased from Povite (Povite, Amsterdam). All other materials were obtained from Merck. Acetrizoate was dissolved in D₂O containing 0.1 M KCl, 0.05 M phosphate buffer, at pH 7.

The relaxation measurements were carried out with a Varian XL-100 instrument equipped with FT facilities. For the T₁ measurements a 180°-t-90° pulse sequence was used. The T₂ relaxation times were determined as T₁ for, using an adiabatic half passage into the center of the resonance line (8). The measurements were conducted at 30-34°C.

RESULTS AND DISCUSSION

A representative example of the nonexponential relaxation (NFR) effect, which can be detected in acetrizoate solutions, is shown in Fig. 1, where the magnetization of the methyl group is plotted as a function of time. Added is also the time dependence of the ring proton magnetization, which is an exponential function of time. The latter observation rules out the possibility that nonlinearities in the detector system are causing the nonexponential time dependence of the methyl group magnetization.

The T₁ relaxation of the methyl group was measured at two different concentrations of acetrizoate (see Table 1). Also the influence of bovine serum albumin (BSA), added
<table>
<thead>
<tr>
<th>Acetazolamide concentration (M)</th>
<th>BSA concentration (M)</th>
<th>Type of relaxation</th>
<th>$T_1$ (a)</th>
<th>$T_2$ (a)</th>
<th>$\delta$</th>
<th>$\sigma$</th>
</tr>
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<tbody>
<tr>
<td>$1 \times 10^{-3}$ M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>$5 \times 10^{-3}$ M</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>$3 \times 10^{-3}$ M</td>
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<td></td>
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<tr>
<td>$5 \times 10^{-3}$ M</td>
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</table>

These experiments were fitted in two ways. The upper values of each experiment were obtained by iteration of $A_i$, $\gamma_i$ and $\delta_i$. The lower values were obtained by direct iteration of $A_i$, $\gamma_i$ and $\delta_i$. $\sigma$ = standard error in calculated values.

Nonexponential Relaxation of Methy1 Protons
to the solutions, was studied. With increasing concentrations of albumin the nonexponential relaxation effect of $T_1$ as well as $T_{1p}$ decreases and at $5 \times 10^{-5} M$ BSA it disappears. This is demonstrated in Fig. 2, where the $T_{1p}$ behavior of the acetrizoate methyl group has been plotted for different BSA concentrations. It is noted in passing that this experiment provides additional proof that the NER effect is not an instrumental artifact.

The effect of the cross-correlation between the motion of the protons within a given methyl group has been extensively studied by Hubbard (1, 2), who showed that in the limit of extreme narrowing the time dependence of the longitudinal as well as the transversal magnetization can be described by two exponentials, i.e.,

$$R(t) = A_1' e^{t/t_1} + A_2' e^{t/t_2},$$

where $R(t) = (\langle I_x \rangle - \langle I_z \rangle^T)/(\cos \theta - 1)\langle I_z \rangle^T$ after a $\theta$ pulse has been applied to the spin system, $\langle I_x \rangle$ stands for the longitudinal magnetization and $\langle I_z \rangle^T$ is its thermal equilibrium value. In case the transversal magnetization time decay is measured $R(t) = \langle I_x \rangle/\sin \theta \langle I_z \rangle^T$ with $\langle I_z \rangle$ being the transversal magnetization. The constants characterizing the relaxation are related by the following expressions

$$A_1' = 1 - A_1,$$  \hfill [2]

and

$$A_1' = \frac{1}{4} \{1 - [4J' + (5/2)J^*][36J'^2 + (45/4)J^*2]^{-1/2}\}$$

Furthermore

$$s_1 = -6J' - (15/2)J^* \pm [36J'^2 + (45/4)J^*2]^{1/2}$$  \hfill [3]
The parameters $J$ and $J'$ are functions of the molecular diffusional constants. When the molecule has arbitrary shape, the expressions for these parameters are quite complicated. If, however $J \ll J'$ it can be deduced from Eqs. [2] and [3] that $A_1' \approx \frac{1}{2}$, $A_2' \approx \frac{3}{2}$ and $|s_1| \ll |s_2|$ with $s_1$ approaching zero. The inequality $J \ll J'$ holds whenever the rotation of the methyl group is rapid with respect to the rotational Brownian motion of the molecule, while the latter motion is supposed to be fast with respect to the Larmor frequency ($2$).

The values of the parameters $v_1$, $v_2$, $A_1$ and $A_2$ in Table 1 (in absence of BSA) and also the curves, drawn in Figs. 1 and 2 (absence of BSA) for the methyl relaxation, were obtained from $J$ and $J'$. The latter two parameters were obtained by fitting the time dependence of the magnetization (Eq [1]) to the experimental points through iteration. When BSA was present in solution, the time dependence of the magnetization was fitted to the experimental points by a direct iteration of $s_1$, $s_2$, $A_1'$ and to their final values assuming no other relationship between $s_1$, $s_2$, $A_1'$ and $A_2'$. The standard errors of the $J'$ and $J''$ and/or $A_1$, $s_1$, $s_2$ are also given in Table 1. In absence of BSA comparison of $A_1$, $s_1$ and $s_2$ with their standard errors demonstrates the significance of the nonexponentiality in the magnetization decay within one experiment. Inspection of Table 1 shows that the values obtained for the acetrizoate methyl group agree within the variation between the experiments with those predicted by Hubbard (2) for a molecule of arbitrary symmetry with $J' \ll J'$. As stated above, these latter parameters are difficult to interpret in terms of molecular rotations. Therefore we estimated the magnitude of the diffusion constants by assuming that the molecular motion was that of a spherical molecule on which the rotation of the methyl group around its axis is superimposed. In that case the three molecular rotational diffusion constants are equal ($D_1$) and the expressions for $J'$ and $J''$ simplify (2) to

$$J' = C/(6D)$$  \[4\]

and

$$J'' = 3C/(v' + 6D)$$  \[5\]

with $v' = 4D_1$, where $D_1$ is the rotational diffusion constant characterizing the rotation of the methyl group around its axis. The constant $C = (3/40)(v^2 h/r^3)$ and $r$ is the distance between the protons in the methyl group. In the calculations below we used $r = 1.80 \, \text{Å}$. For the values of $J'$ and $J''$ given in Table 1, $D_1$ and $D$ were calculated and tabulated in Table 2. As expected the rotation of the methyl group around its axis is indeed much faster (about 30 times) than the rotation of the whole molecule. However, the value of

<table>
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<th>TABLE 2</th>
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<td>Rotational Diffusion Constants</td>
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</tbody>
</table>

<table>
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<tr>
<th>Acetrizoate concentration</th>
<th>$D$ rad$^2$ sec$^{-1}$</th>
<th>$D_1$ rad$^2$ sec$^{-1}$</th>
<th>Type of relaxation</th>
</tr>
</thead>
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<tr>
<td>$1 \times 10^{-3} , M$</td>
<td>$1.5 \times 10^9$</td>
<td>$58 \times 10^9$</td>
<td>$T_1$</td>
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<tr>
<td>$5 \times 10^{-3}$</td>
<td>$2.3 \times 10^9$</td>
<td>$77 \times 10^9$</td>
<td>$T_{1p}$</td>
</tr>
<tr>
<td>$5 \times 10^{-3}$</td>
<td>$2.2 \times 10^9$</td>
<td>$34 \times 10^9$</td>
<td>$T_{1p}$</td>
</tr>
</tbody>
</table>
Recently Werbelow and Marshall (4) extended Hubbard's theory to the case where the limit of extreme narrowing is no longer valid. In this situation the time dependence of the magnetization is described by the sum of three exponentials:

\[ R(t) = A_1 e^{t} + A_2 e^{t} + A_3 e^{t} \]  

For a symmetric top molecule, with the axis of the methyl group along the molecular symmetry axis, the authors designed contour plots exhibiting the preexponential factors \( A_i \) and the ratios of the time constants \( \lambda_i/\lambda_j \) as a function of the rotational diffusion constants.

More detailed plots of the longitudinal relaxation parameters for regions of the rotational diffusion coefficients relevant to the present problem, were constructed and are given in Fig. 3. These show some interesting features.

When \( D \) has a value characteristic of the extreme narrowing limit and \( D_1 + D_2 \) \( \approx D \), where \( D_1 \) is the diffusion constant parallel and \( D_2 \) that perpendicular to the molecular symmetry axis, the longitudinal relaxation parameters take the following simple form: \( A_1 = \frac{1}{2} ; A_2 = \frac{1}{2} ; A_3 = \frac{1}{2} \) and \( |\lambda_1| \leq |\lambda_2| \) with \( \lambda_1 \) approaching zero. These results are obtained even when \( D_1 \) is not representative of the extreme narrowing limit. When it is, but remains sufficiently small with respect to \( D_1 + D_2 \), the time constant \( \lambda_2 \approx \lambda_3 \) and we obtain the same constants as were calculated by Hubbard for a molecule of arbitrary symmetry with \( J'' \ll J' \), discussed above, so that \( A_1' = A_1 ; A_2' = A_2 + A_3 ; s_1 = \lambda_1 \) and \( s_2 = \lambda_2 = \lambda_3 \). In the Appendix it will be shown that these observations can be cast into an analytical form. Independently of Hubbard's model, the first three experiments in Table 1 can be fitted with a two-exponential function and this yields preexponential factors and time constants which are close to those obtained by fitting \( J' \) and \( J'' \). Using the average values of these first three experiments in combination with Fig. 3, we estimate \( (D_1 + D_2) = 7 \times 10^{10} \text{ rad}^2 \text{ sec}^{-1} \) and \( D_1 = 2 \times 10^9 \text{ rad}^2 \text{ sec}^{-1} \). Thus both models give diffusion constants in reasonable agreement with each other, while \( A_2' = A_2 + A_3 \) with \( A_2 = 0.3 \) and \( A_3 = 0.6 \). On the other hand, taking the radius of the iodobenzene group equal to 5.6 Å yields a value of the rotational diffusion constant, characteristic of the rotation of the whole molecule, of \( D = 9.3 \times 10^{10} \text{ rad}^2 \text{ sec}^{-1} \), calculated on the basis of the Stokes-Einstein model. This again is close to the values given in Table 2. Thus it is anticipated that the methyl group relaxation of molecules with a radius of about 6 Å and higher (see Appendix), will be strongly nonexponential, provided that spin-rotation and intermolecular relaxation contributions are not important and the rotation of the methyl group is not hindered too strongly. In the present experiments the intermolecular relaxation was suppressed by carrying out the measurements in \( D_2O \) and the spin rotation contributions were estimated to be smaller than 1% (9).

In the presence of BSA the NLR effect disappears (see Fig. 2 and Table 1). Several mechanisms may be responsible for this result. If the rotation of the methyl group is not

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**Fig. 3** Contour plots of the preexponential factors \( A_i \) and the exponentials \( \lambda_i \) of the longitudinal relaxation as a function of \( D \) and \( (D_1 + D_2) \). Each contour is a line of constant magnitude of the indicated relaxation parameter. The calculations have been performed for \( \omega_0 = 2\pi \times 10^8 \) as the Larmor precession frequency.
Fig. 3.
affected upon binding of acetrizoate to the protein molecule, one would expect the relaxation to remain strongly nonexponential (see Appendix). If, on the other hand, the rotation of the methyl group is immobilized to the same extent as the acetrizoate molecule, the NER effect may disappear. Also protons of the BSA molecule may contribute to the relaxation of the methyl protons and obscure the nonexponential behavior. Most probably, however, chemical exchange terms will dominate the relaxation and mask the NER effect. The binding of triognost to BSA is rather involved, because of the existence of several binding sites with different binding constants (10) and therefore we shall not elaborate on this any further.

APPENDIX

The differential equations determining the time dependence of the longitudinal magnetization of a spin system of three identical spin-½ nuclei at the corners of an equilateral triangle are given by (1):

\[
d y_1/dt = -[2J_a(\omega_0) + 8J_a(2\omega_0) + 2J_c(\omega_0)]y_1(t) \\
+ [-12J_c(\omega_0) + 24J_c(2\omega_0)]y_2(t) \\
+ 16[J_c(\omega_0) - J_c(2\omega_0)]y_3(t), \\
\]

\[[A1]\]

\[
d y_2/dt = (\frac{1}{3})[J_a(\omega_0) - 4J_a(2\omega_0) - J_c(\omega_0) + 4J_c(2\omega_0)]y_1(t) \\
- [3J_a(0) - J_a(\omega_0) + 2J_a(2\omega_0) - 3J_c(0) + J_c(\omega_0) - 2J_c(2\omega_0)]y_2(t) \\
+ (\frac{1}{3})[6J_a(0) - 10J_a(\omega_0) + 4J_a(2\omega_0) - 6J_c(0) + 10J_c(\omega_0) \\
- 4J_c(2\omega_0)]y_3(t), \\
\]

\[[A2]\]

\[
d y_3/dt = [J_a(\omega_0) - 2J_a(2\omega_0) + J_c(\omega_0)]y_1(t) \\
+ (\frac{1}{3})[J_a(\omega_0) + 3J_c(\omega_0) + 12J_c(2\omega_0)]y_2(t) \\
- [9J_a(\omega_0) - J_c(\omega_0) + 4J_c(2\omega_0)]y_3(t), \\
\]

\[[A3]\]

where \( y_1(t) = \text{Tr}[\chi(t)I_z] = \langle I_z \rangle - \langle I_z \rangle^T \); \( \langle I_z \rangle \) is the longitudinal magnetization and \( \langle I_z \rangle^T \) the thermal equilibrium value of \( I_z \), while \( \chi(t) \) represents the appropriate density matrix. \( y_2(t) \) and \( y_3(t) \) are the other combinations of matrix elements (see (1) and (4)). \( J_a(\omega_0) \) is the spectral density of the autocorrelation function and \( J_c(\omega_0) \) that of the cross-correlation function. Hubbard showed (1) that in the limit of extreme narrowing, Eqs. [A1] and [A2] are no longer coupled to [A3]. The system of differential equations also considerably simplifies when \( J_a(\omega_0) = J_c(\omega_0) = I(\omega_0) \). In this case Eq. [A2] reduces to \( dy_2/dt = 0 \) and the time dependence of the longitudinal magnetization is found to be:

\[
R(t) = \frac{1}{6} + \frac{1}{6}e^{-12I(\omega_0)t} + \frac{1}{6}e^{-12I(2\omega_0)t}. \\
\]

\[[A4]\]

Using the differential equations characterizing the transversal magnetization (4) we obtain, under the conditions that \( J_a(\omega_0) = J_c(\omega_0) = I(\omega_0) \), for the time dependence of the transversal magnetization:

\[
Q(t) = \frac{1}{6} + \frac{1}{6}e^{-6I(0) + I(\omega_0)t} + \frac{1}{6}e^{-6I(\omega_0) + I(2\omega_0)t}. \\
\]

\[[A5]\]

In the limit of extreme narrowing \( I(0) = I(\omega_0) = I(2\omega_0) \) and as expected [A4] and [A5] become equal and the time dependence of the longitudinal and transversal relaxation
reduces to that described by Hubbard (2), i.e., the parameters of Eq. [1] become $A'_1 = \frac{1}{2}, A'_2 = \frac{3}{8}$ and $s_1 = 0$.

In general it is not easy to show under which circumstances $J_\alpha(k\omega_0) = J_\varepsilon(k\omega_0)$. For a symmetric top molecule, however, with the axis of the methyl group along the symmetry axis of the molecule, the following relations can be derived (4):

$$J_\alpha(k\omega_0) = C\left\{ \frac{6D_{\perp}}{(6D_{\perp})^2 + (k\omega_0)^2} + \frac{3[2D_{\perp} + 4(D_1 + D_2)]}{[2D_{\perp} + 4(D_1 + D_2)]^2 + (k\omega_0)^2} \right\}$$  \[A6\]

and

$$J_\varepsilon(k\omega_0) = C\left\{ \frac{6D_{\perp}}{(6D_{\perp})^2 + (k\omega_0)^2} - \frac{1}{2} \frac{3[2D_{\perp} + 4(D_1 + D_2)]}{[2D_{\perp} + 4(D_1 + D_2)]^2 + (k\omega_0)^2} \right\}$$  \[A7\]

If the second term within the brackets, $"(k\omega_0)/C$ in [A6] and $-\frac{1}{2}"(k\omega_0)/C$ in [A7], can be neglected with respect to the first term, $'(k\omega_0), J_\alpha(k\omega_0)$ equals $J_\varepsilon(k\omega_0)$. This condition is met, when the following relations hold.

(i) $(D_1 + D_2) \gg D_{\perp}, (k\omega_0),$

(ii) $(D_1 + D_2) D_{\perp} \gg (k\omega_0)^2$.

The present analysis shows that whenever the rotation of the methyl group around its axis is in the extreme narrowing limit and much faster than the rotation of the molecule perpendicular to this axis, the methyl group relaxation can be described by Eqs [A4] and [A5]. These even apply when the molecular rotation, characterized by $D_{\perp}$, is not in the extreme narrowing limit. There is, however, a lower limit to $D_{\perp}$ given by condition (ii).

Expressions [A4] and [A5] are of practical importance for protein and membrane systems, provided the rotation of the methyl group is relatively unhindered and the rotation of the molecule is relatively slow, since proteins often are globular in nature and the effective motion of molecules in a membrane often resembles that of a symmetric top molecule.

Interestingly the initial slopes of $R(t)$ and $Q(t)$ (Eqs [A4] and [A5]) are given by

$$dR(0)/dt = -2I(0) - 8I(2\omega_0)$$

and

$$dQ(0)/dt = -3I(0) - 5I(\omega_0) - 2I(2\omega_0).$$

Since $I(k\omega_0) = J_\alpha(k\omega_0)$ these slopes are just equal to those obtained when the influence of cross-correlation is disregarded (11).

ACKNOWLEDGMENT

It is a pleasure to acknowledge the assistance of Th de Boo and R de Graaf of the Department of Statistical Consultation in the numerical and analytical calculations.

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A NMR STUDY OF THE KINETICS OF THE BINDING OF
THE RENAL CONTRAST MEDIUM ACETRIZOATE TO ALBUMIN

J.F. Rodrigues de Miranda† and C.W. Hilbers‡
University of Nijmegen, Nijmegen, The Netherlands.

†Department of Pharmacology
‡Department of Biophysical Chemistry
Protein binding of drugs, in particular drug-albumin binding, can interfere with a number of biophysical processes in the body (1, 2, 3). It may for instance have a profound influence on the pharmacokinetics of the drug involved. Two factors are important in this respect. First the amount of drug bound to the protein, determined by the affinity constant and the concentrations of protein and drug, since albumin-bound drug is not susceptible to ultra filtration and to diffusion processes occurring for instance in kidney, liver, brain, etc. (4, 5). Moreover, in general the fraction of bound drug will not be directly involved in drug action (2). On the other hand the drug-protein complex can serve as a drug depot i.e. release of the drug from the complex will tend to maintain the free drug concentration (4, 6). This constitutes the second factor of importance, the dissociation rate of the drug-protein complex, which may affect the active transport processes e.g. in kidney, liver and brain.

Dynamic events occurring during protein-drug binding can be studied by magnetic resonance techniques. Exchange of the drug molecules between protein and solution may cause shifting and broadening of the nmr signals (7, 8, 9). The linebroadening and shifts are determined by three independent parameters: i) the relaxation time of the drug molecule when bound to the protein, ii) the exchange rate and iii) the difference between resonance frequencies of the protons of the drug molecule when bound to the protein or free in solution.

In the present paper the binding of the renal contrast medium acetrizoate (Triognost) to bovine serum albumin has been studied by proton magnetic resonance. In relation to its renal tubular excretion it is of interest to know whether the dissociation rate of the acetrizoate-albumin complex is rate limiting in the active excretion process (10, 11). Moreover, the strong binding of acetrizoate to albumin compared to the weak binding of other contrast media of the tri-iodo-benzoic acid family (10) leads one to wonder as to what part or group of the acetrizoate molecule may be responsible for the rather specific and strong binding of acetrizoate to albumin.

Acetrizoate seemed to be particularly suited for a nmr study because of the simplicity of its nmr spectrum and because according
to Lang and Lasser (12) binding predominantly takes place at one site on the albumin molecule. During the course of this investigation it became evident from nmr as well as from independent dialysis binding studies that there are at least two groups of binding sites on the albumin molecule. In spite of this complication the dissociation rate of the different type of complexes could be estimated by combining nmr and equilibrium dialysis experiments. It can also be derived from the present and other investigations that the interpretation of earlier nmr studies of pharmacologically interesting systems may be over­simplified.

EXPERIMENTAL

The acetrizoate (Triognost \textsuperscript{R}) used in the nmr as well as the dialysis experiments and the starting material (2,4,6-tri-iodo-3-amino benzoic acid) for the C\textsuperscript{14}-labelled acetrizoate were kindly supplied by Dagra (Dagra, Diemen, The Netherlands). Bovine serum albumin (BSA) was purchased from Povite (Povite, Amsterdam). All other materials were obtained from Merck, Darmstadt, Germany.

![Acetrizoate](image)

Acetrizoate-C\textsuperscript{14} was synthesized by adding 40 mg of 2,4,6-tri-iodo-3-amino benzoic acid to 4 mg acetic anhydride-C\textsuperscript{14}, which had been dissolved in 5 ml acetonitrile under exclusion of air. The acetonitrile was freshly distilled from P\textsubscript{2}O\textsubscript{5}; SO\textsubscript{3} was used as a catalyst. The radiochemical purity was checked by taking an autoradiogram of a kieselgel dunnschicht chromatogram, developed in a solution of benzene, ethanol, acetic acid in the ratio 7:1:2.

The binding of acetrizoate to albumin was determined in equilibrium dialysis experiments at 4\textdegree{} and 30\textdegree{}C by measuring the radioacti-
vity of the acetrizoate-C\textsuperscript{14}, present in tracer amounts in the samples, on both sides of the membrane (13, 14). The samples were prepared by dissolving the required amounts of acetrizoate and albumin in H\textsubscript{2}O containing 0.1 M KCl, 0.05 M phosphate buffer, while at pH 7. The nmr samples were prepared in D\textsubscript{2}O, the solutions were of the same composition and pH as those used in the dialysis experiments.

The nmr experiments were carried out on a Varian XL-100 instrument equipped with F.T. facilities. The spectra were obtained either by accumulation in a Varian C1024 time average computer or by accumulation using the F.T. technique. In the latter case a (180\textsuperscript{0}-\textit{t}-90\textsuperscript{0}-\textit{T}) pulse sequence was used in order to avoid dynamic range problems, connected with the limited wordlength of the computer memory, due to the residual HDO resonance (15). Samples were freshly prepared just prior to the measurements.

**THEORETICAL BACKGROUND**

As will be shown in the next section to a good approximation the binding of acetrizoate to BSA can be described by two classes of independent identical sites on the albumin molecule. Exchange of the acetrizoate molecules bound to the protein and free in solution results in a transfer of magnetization between three different environments. The transition probabilities between these states can be defined as follows:

\[
\begin{align*}
\tau^{-1}_\text{IS} &= k^+_I ; \\
\tau^{-1}_\text{SI} &= k^+_I \sum_{j=1}^{N} (\text{PS}_{j-1})_I \\
&\quad \text{all configurations} \\
\tau^{-1}_\text{IIS} &= k^+_I ; \\
\tau^{-1}_\text{SI} &= k^+_I \sum_{j=1}^{N} (\text{PS}_{j-1})_{II} \\
&\quad \text{all configurations}
\end{align*}
\]

where \(k^+_I\) and \(k^+_I\) are the dissociation rate constants for the complexes.
of the type I and II and $k_{I}^{+}$ and $k_{II}^{+}$ are the corresponding association rate constants, defined by the following reaction equation:

$$PS_{j-1} + S \xrightleftharpoons[k^{-}]^{k^{+}} PS_{j}$$

(2)

$PS_{j}$ is a particular configuration of $j$ molecules $S$ bound to a certain class of binding sites on the protein molecule. The $\sum$ signs indicate a summation over all particles $PS_{j}$ and over all their configurations present in solution. The definitions in eqs. 1 implicitly assume that binding to the sites takes place independently and that the sites within a class are identical. Moreover, it is assumed that no direct exchange takes place between the binding sites of class I and II.

The dissociation and association transition probabilities are related by the following equation:

$$\frac{1}{\tau_{S_i}^{-1}} = \frac{P_{0}}{[S]} \bar{r}_{i} \frac{1}{\tau_{iS}}$$

(3)

where $\bar{r}_{i}$ represents the average number of acetrizoate molecules bound per albumin molecule for a particular class, $i$, of binding sites; $P_{0}$ is the concentration of protein weighed into the sample and $[S]$ is the concentration of free substrate molecules. Within the approximations mentioned above i.e. independent and identical binding sites, the average number of molecules bound per albumin molecule for particular class of binding sites is given by (13, 16)

$$\bar{r}_{i} = \frac{n_{i}K_{i}[S]}{1 + K_{i}[S]}$$

(4)

where $K_{i}$ is the equilibrium constant characterizing reaction equation 2 and $n_{i}$ is the total number of sites of the class $i$ available for binding. The total average number of acetrizoate molecules bound to
BSA is:

\[
\frac{1}{T_2} = \frac{1}{T_2} + \frac{1}{T_{2s}} = \frac{n_1 k_{IS} [S]}{1 + K_{IS} [S]} + \frac{n_{II} k_{II} [S]}{1 + K_{II} [S]}
\]  (5)

The nmr experiments were carried out with a large excess of substrate molecules so that using the eq. 3, the dissociation rates can be directly related to the observed linebroadening (17):

\[
\frac{1}{T_2} - \frac{1}{T_{2s}} = \frac{P_0}{S_0} \left\{ \frac{1}{T_{II} X_{II}} + \frac{1}{T_{II} X_{II}} \right\}
\]  (6)

where \( T_2^{-1} \) equals \( n \Delta \nu \) with \( \Delta \nu \) being the observed linewidth at half-height of the substrate resonances in the presence of protein. \( T_{2s}^{-1} \) is the reciprocal transversal relaxation time of the substrate resonance in the absence of protein. \( S_0 \) is the concentration of substrate weighed into the sample. The linebroadening parameters \( X_I \) and \( X_{II} \) are concentration independent. They are a function of the dissociation rate constant, \( r_{IS}^{-1} \), the relaxation rate of the nucleus in the substrate molecule while bound to the protein molecule, \( T_{2i}^{-1} \), and the chemical shift difference \( \Delta \omega_i \) between the resonance position free in solution and bound to one of the sites of class \( i \), i.e. (17, 18):

\[
X_i = \frac{\tau_{IS}^{-1} \left( \tau_{IS} T_{2i}^{-1} (1 + \tau_{IS} T_{2i}^{-1}) + (\Delta \omega_i \tau_{IS})^2 \right)}{(1 + \tau_{IS} T_{2i}^{-1})^2 + (\Delta \omega_i \tau_{IS})^2}
\]  (7)

In an analogous fashion the observed changes in the resonance positions are related to the binding parameters by (17):

\[
\delta = \frac{P_0}{S_0} \left\{ \frac{1}{T_{II} X_{II}} + \frac{1}{T_{II} X_{II}} \right\}
\]  (8)
\[
\Delta_i = \frac{\Delta\omega_i}{(1 + \tau_{iS} T_{2i}^{-1})^2 + (\Delta\omega_i \tau_{iS})^2}
\]  

(9)

In practice expressions 7 and 9 can often be simplified. Recently Granot and Fiat (19) have systematically reviewed the possible simplifications of eqs. 7 and 9. These results will not be reproduced here, but for the readers convenience those limiting cases employed in the following sections will be given.

Case I:

Complexation of acetrizoate to the specific binding site on albumin resulted in zero values for \(\Delta_i\) (see Results Section). Under these circumstances the following conditions apply:

\[
\Delta\omega_i \ll \tau_{iS}^{-1} \quad \text{or} \quad \Delta\omega_i \approx 0
\]

i.e. equations 7 and 9 can be simplified to:

\[
X_i = (\tau_{iS} + T_{2i})^{-1}
\]

(10)

and

\[
\Delta_i = \Delta\omega_i \tau_{iS}^{-2} (\tau_{iS}^{-1} + T_{2i}^{-1})^{-2}
\]

(11)

\(\Delta_i\) approaches zero when \(\Delta\omega_i\) approaches zero and/or \(\tau_{iS}\) is much larger than \(T_{2i}\).

Case II:

For the binding of acetruziate to the second class of binding sites we have no clue as to the relative values of \(T_{2i}^{-1}\) and \(\Delta\omega_i\). We
will therefore start out to assume that $\Delta \omega_i \gg T_{2i}^{-1}$. Since the temperature dependence of the linebroadening indicates the existence of a fast exchange situation i.e. $(\Delta \omega_i \tau_{is})^2 \ll 1$, eqs. 7 and 9 can be written as:

$$X_i = (\tau_{is} + T_{2i})^{-1} + \Delta \omega_i^2 \tau_{is} (1 + \tau_{is} T_{2i}^{-1})^{-2}$$

and

$$\Delta_i = \Delta \omega_i (1 + \tau_{is} T_{2i}^{-1})^{-2}$$

Since in the present circumstances $(\tau_{is} T_{2i}^{-1})^2 \ll 1$, these equations can be simplified to:

$$X_i = (\Delta \omega_i + \Delta)(2T_2 \Delta \omega_i)^{-1} + \tau_{is} \Delta_i \Delta \omega_i$$

and

$$\Delta_i = \Delta \omega_i (1 - 2\tau_{is} T_{2i}^{-1})$$

Fast exchange conditions applied to the limiting cases where $T_{2i}^{-1} \ll \Delta \omega_i$ or $T_{2i}^{-1} \gg \Delta \omega_i$ lead to formulae, which are not compatible with the observed linebroadenings and shifts.

RESULTS

A. CONCENTRATION DEPENDENCE

An example of the linebroadening observed for the acetrizoate resonances as a result of binding to albumin is shown in Fig. 1. The lower half of the figure displays the spectrum of a $6 \times 10^{-3}$ M ace-
trizoate solution without albumin, the upper half of the figure was recorded with $2.4 \times 10^{-5}$ M albumin present in the sample.

![NMR spectra](image)

Fig. 1. NMR spectra of acetrizoate ($6 \times 10^{-3}$ M) in a $D_2O$-phosphate buffer, pH = 7.4. Lower spectrum without, upper spectrum with albumin ($2.4 \times 10^{-5}$ M). The resonances at 58, 160, 260 and 780 Hz were recorded on an expanded scale and at a higher amplification factor and inserted at their corresponding frequencies.

The intense signal at about 430 Hz is due to residual HDO. The resonances of interest, at 58, 160, 260 and 780 Hz respectively, were recorded on an expanded scale and at a higher amplification factor and were inserted in the figure at their corresponding frequencies. Proceeding from left to right they represent the $\phi$-H of acetrizoate, the methylgroups of tetramethylammoniumchloride (TMAC), the methylgroup of acetrizoate and the methylgroup of t-butylalcohol. The methylresonances of tetramethylammoniumchloride and t-butylalcohol served as a check on the field homogeneity and as internal references. Addition of albumin clearly shows a linebroadening of the acetrizoate resonances while the
reference signals remain virtually unchanged. For a series of solutions with constant acetrizoate concentration, $S_0$, but increasing BSA concentration, $P_0$, the linewidths were measured. Chemical shift changes, $\delta$, were determined with respect to TMAC. In all samples acetrizoate was present in large excess. The data thus obtained were plotted as a function of $P_0/S_0$. In Fig. 2 such a plot of $T_2^{-1}$ as function of $P_0/S_0$ is presented, for the $\text{CH}_3$ and $\phi$-$\text{H}$ resonances of acetrizoate, while in Fig. 3 analogous plots of $\delta$ are given.

Fig. 2. Plot of $T_2^{-1}$ of the $\phi$-$\text{H}$ and $\text{CH}_3$ protons of acetrizoate as a function of the ratio of the amount of protein and substrate weighed into the sample. The acetrizoate concentration was kept constant at $3.5 \times 10^{-3}$ M. Each point is an average of at least six independent linewidth measurements. Temperature $30^\circ\text{C}$. The solid lines represent least squares fits to the experimental points based on equation 6.
Fig. 3. Plot of chemical shift values, $\delta$, of the $\Phi$-H and CH$_3$ protons of acetrizoate as a function of the ratio of the amount of protein and substrate weighed into the sample. Each point is an average of at least six independent shift measurements. Acetrizoate concentration $3.5 \times 10^{-3}$ M. Temperature $30^\circ$C. The solid lines represent least squares fits to the experimental points based on equation 8.

As expected on the basis of eqs. 6 and 8, $T_2^{-1}$ and $\delta$ linearly increase with increasing BSA concentration.

Before an interpretation of the slope of the lines in Fig. 2 in terms of exchange and relaxation phenomena can be given, it is necessary to show whether the broadening is due to specific or multiple binding. To this end a series of experiments, like those given in Fig. 2, were conducted with varying acetrizoate concentrations. The slopes of the lines, $\tan \alpha$, obtained in the individual experiments, turned out to be a function of the acetrizoate concentration. This points to the existence of binding sites on the albumin molecule which are not yet
occupied in the acetrizoate concentration range studied in the nmr experiments. In view of the data of Lang and Lasser (12) this result was somewhat unexpected and therefore a series of equilibrium dialysis experiments were carried out.

The results of these experiments conducted at 4°C are given in Fig. 4, where the average total number of acetrizoate molecules, \( \bar{n}_T \), bound per albumin molecule has been plotted against the logarithm of acetrizoate concentration free in solution.

![Fig. 4. The average total number of acetrizoate molecules, \( \bar{n}_T \), bound per albumin molecule plotted as a function of the acetrizoate concentration free in solution. The points were obtained from equilibrium dialysis experiments conducted at 4°C. The solid line is a least square fit to the experimental values based on equation 5.](image)

The points show that in a concentration region around \( 10^{-6} \) M a first binding site is titrated. A second set of sites is titrated at much higher concentrations namely from \( 10^{-3} \) to \( 10^{-2} \) M and does not show any saturation up to the highest concentration studied. On the assumption that two different classes of binding sites are present on the albumin molecule a curve was fitted to the experimental points (solid line in Fig. 4) using equation 5 to describe the binding. To this end a computer fitting procedure was employed based on a gradient method for the
nonlinear parameter $K$ and a linear regression method for the linear parameter $n$. The resulting binding parameters together with their standard errors are given in table 1 for two series of experiments, i.e. at $4^\circ$ and $30^\circ$C.

Table 1

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Class I</th>
<th>Class II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$n_I$</td>
<td>$k_I \times 10^{-5} \text{ M}^{-1}$</td>
</tr>
<tr>
<td>$4^\circ$C</td>
<td>0.85 (.02)</td>
<td>16.0 (.8)</td>
</tr>
<tr>
<td>$30^\circ$C</td>
<td>0.98 (.02)</td>
<td>4.6 (.2)</td>
</tr>
</tbody>
</table>

The values in the parentheses are the standard errors.

From Fig. 4 it can be concluded that in the concentration range in which the nmr experiments were conducted ($10^{-3} - 2 \times 10^{-2} \text{ M}$) there are at least two types of complexes present in solution. Moreover, in this concentration range, the first class of binding sites is saturated, i.e. $r_I = 0.9$ throughout the concentration range of acetrizoate, explored in the nmr experiments. The values of $\tau \alpha$ obtained at $4^\circ$ and $30^\circ$C are summarized in table 2 together with the experimentally determined values of $r_T$. The chemical shift data have been evaluated in an analogous fashion i.e. the slopes of the lines, $\tau \beta$, obtained by plotting $\delta$ versus $P_0/S_0$ (see Fig. 3) were determined for different concentrations of acetrizoate and are listed in table 2.

B. EVALUATION OF THE LINEBROADENING PARAMETERS

According to eq. 6

$$\tau \alpha = r_I X_I + r_{II} X_{II}$$ (16)
Table 2
Linewidth and chemical shift parameters of the acetrizoate-BSA complex

<table>
<thead>
<tr>
<th>$S_0$ mM $^a)$</th>
<th>$\bar{r}_T$ b)</th>
<th>Linewidth $^c)$</th>
<th>Chemical shift $^c)$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$\text{tg } \alpha (\phi-H)$</td>
<td>$\text{tg } \alpha (\text{CH}_3)$</td>
</tr>
<tr>
<td>$30^\circ C$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>2.0</td>
<td>93 (3)</td>
<td>102 (8)</td>
</tr>
<tr>
<td>2.0</td>
<td>2.8</td>
<td>157 (16)</td>
<td>146 (10)</td>
</tr>
<tr>
<td>3.5</td>
<td>3.7</td>
<td>207 (18)</td>
<td>219 (15)</td>
</tr>
<tr>
<td>6.0</td>
<td>4.7</td>
<td>282 (20)</td>
<td>265 (12)</td>
</tr>
<tr>
<td>10.0</td>
<td>6.1</td>
<td>376 (28)</td>
<td>318 (11)</td>
</tr>
<tr>
<td>15.0</td>
<td>7.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20.0</td>
<td>8.7</td>
<td>609 (22)</td>
<td>516 (30)</td>
</tr>
<tr>
<td>$4^\circ C$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>1.7</td>
<td>85 (4)</td>
<td>114 (9)</td>
</tr>
<tr>
<td>2.0</td>
<td>2.2</td>
<td>179 (10)</td>
<td>134 (18)</td>
</tr>
<tr>
<td>4.0</td>
<td>3.1</td>
<td>234 (15)</td>
<td>216 (42)</td>
</tr>
<tr>
<td>6.5</td>
<td>4.0</td>
<td>485 (22)</td>
<td>393 (25)</td>
</tr>
<tr>
<td>10.0</td>
<td>5.1</td>
<td>565 (11)</td>
<td>457 (41)</td>
</tr>
<tr>
<td>18.4</td>
<td>7.0</td>
<td>896 (13)</td>
<td>651 (39)</td>
</tr>
</tbody>
</table>

$^a)$ $S_0$ is the total concentration of acetrizoate

$^b)$ $\bar{r}_T$ is the total average number of acetrizoate molecules bound per albumin molecule

$^c)$ The values in the parentheses are standard errors

The binding experiments (see Fig. 4) have shown that in all nmr experiments $\bar{r}_I = 0.9$, so that:

$$\text{tg } \alpha = 0.9 \ X_I + (\bar{r}_T - 0.9) \ X_{II} \quad (17)$$

Plotting $\text{tg } \alpha$ versus $(\bar{r}_T - 0.9)$ should yield a straight line with a slope given by $X_{II}$ and an intercept by 0.9 $X_I$. The experimental results
obtained at 4° and 30°C are given in Fig. 5 for both the CH₃ and the φ-H protons.

Fig. 5. Plot of \( \tan \alpha \) versus the average number of acetrizoate molecules bound to the second class of binding sites, \( \bar{r}_M - \bar{r}_T \). Each point is the slope of a line representing the \( T_0 \) dependence on \( P_0/S_0 \), of which examples are given in Fig. 2. The solid lines are least squares fits based on equation 17.

A least squares fit to the points results in the values for \( X_I \) and \( X_{II} \) given in table 3.

It should be noted in passing that independently of the interpretation of the binding curve (Fig. 4) the nmr data are inconsistent with the assumption of one class of binding sites for acetrizoate on the albumin molecule. If albumin were to possess only one class of independent and identical binding sites, the linebroadening parameter \( \tan \alpha/\bar{r}_T \) would be concentration independent.
Table 3
Linebroadening (X) and chemical shift (Δ) parameters of the acetrizoate-BSA complex

<table>
<thead>
<tr>
<th>class of binding sites</th>
<th>group</th>
<th>X (sec⁻¹)</th>
<th>Δ (sec⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4°C</td>
<td>30°C</td>
</tr>
<tr>
<td>I</td>
<td>CH₃</td>
<td>27 (20)</td>
<td>43 (10)</td>
</tr>
<tr>
<td></td>
<td>φ-H</td>
<td>0 (20)</td>
<td>15 (10)</td>
</tr>
<tr>
<td>II</td>
<td>CH₃</td>
<td>100 (12)</td>
<td>59 (3)</td>
</tr>
<tr>
<td></td>
<td>φ-H</td>
<td>141 (14)</td>
<td>72 (4)</td>
</tr>
</tbody>
</table>

Values in the parentheses are standard errors.

Examination of Fig. 6, in which $\frac{\alpha}{r_T}$ of the phenylprotons, obtained at 30°C, has been plotted as a function of $S_0$, clearly reveals a concentration dependence, which is compatible only with the existence of more than one class of binding sites.

![Graph of $\frac{\alpha}{r_T}$ versus $S_0$ for the φ-H proton. Temperature 30°C.](image)

Fig. 6. Plot of $\frac{\alpha}{r_T}$ versus $S_0$ for the φ-H proton. Temperature 30°C. The solid line has been calculated according to equation 18 (see text).
The line drawn through the points has been calculated using the expression:

$$\tan \alpha / \bar{T} = 0.9 X_I + (\bar{T} - 0.9) X_{II} / \bar{T}$$  \hspace{1cm} (18)$$

The \( \bar{T} \) values were taken from Fig. 4 and \( X_I \) and \( X_{II} \) from table 3.

Except for class I at 4°C, where the standard errors in the line-broadening parameters are too large, it can be concluded that the values of \( X_I \) for the aromatic and methyl protons are significantly different from each other for both classes of binding sites (see table 3).

**Fig. 7.** Plot of \( \tan \beta \) as a function of the average number of acetazolamide molecules bound to the second class of binding sites \( \bar{P}_T - \bar{P}_I \). Each point is the slope of a line representing the \( \delta \) dependence on \( P_0/S_0 \), of which examples are given in Fig. 3. The solid lines are least squares fits based on equation 19.

C. EVALUATION OF THE SHIFT PARAMETERS

According to equation 8 \( \tan \beta \) is related to the chemical shift parameter \( \Delta_I \) as:
\[ \tan \beta = 0.9 \Delta_I + (\bar{r}_T - 0.9) \Delta_{II} \]  \hspace{1cm} (19)

Plotting \( \tan \beta \) versus \( (\bar{r}_T - 0.9) \) should yield a straight line with a slope given by \( \Delta_{II} \) and an intercept by \( 0.9 \Delta_I \). The experimental results obtained at \( 4^\circ \) and \( 30^\circ \)C are given in Fig. 7 for both the CH\(_3\) and the \( \phi\)-H protons. A least square fit to the points results in values for \( \Delta_I \) and \( \Delta_{II} \) given in table 3. Examination of this data shows that the shift parameters \( \Delta_I \) of the first class of binding sites are not different from zero within experimental error for both the CH\(_3\) and the \( \phi\)-H protons. On the other hand the \( \Delta_{II} \) values of the second class of binding sites do differ appreciably from zero. Moreover, these values decrease significantly with decreasing temperature.

D. DETERMINATION OF THE DISSOCIATION AND RELAXATION RATES

As indicated above no chemical shift changes occur upon binding of acetrizoate to the specific binding site (class I) of BSA. Consequently equation 10 will be used to interpret the linebroadening parameters. At \( 30^\circ \)C the \( X_I \) values of the phenyl and methyl protons are significantly different (see table 3). Considering equation 10 this inequivalence is indicative of a situation in which only the relaxation rate, \( T_{2I}^{-1} \), or both the exchange rate, \( r_{IS}^{-1} \), and the relaxation rate, \( T_{2I}^{-1} \), contribute to the value of \( X_I \). If the lifetime of the albumin acetrizoate complex were much longer than the transversal relaxation times \( T_{2I} \) of the acetrizoate protons when bound to the albumin, the linebroadening parameters \( X_I \) for the \( \phi\)-H and CH\(_3\) protons should be equal, contrary to what is observed.

The lifetime of the acetrizoate - BSA complex of the type I is estimated as follows: Firstly from equation 10 and the \( X_I \) values at \( 30^\circ \)C (table 3) it immediately follows that \( \tau < 0.025 \text{ sec.} \) Secondly by making a reasonable estimate of the upper limit of \( T_{2I}^{-1} (\text{CH}_3) \), a lower limit of the lifetime of the specific acetrizoate albumin complex can be computed. The \( T_{2I}^{-1} (\text{CH}_3) \) value can be derived from Fig. 1 of a paper of Werbelow and Marshall (20). To this end we assume that the acetrizoate molecule is immobilized on the protein surface, while the methyl group...
retains the motional freedom around its axis. Taking the rotational correlation time, $\tau_c$, of BSA equal to $10^{-8}$ sec (21) and the rotational correlation time $\tau_{\text{int}}$ for the methyl group rotation around its axis equal to $0.5 \times 10^{-11}$ sec (22) we find $T_{2I} (\text{CH}_3) = 10^{-2}$ sec. This estimate of $T_{2I} (\text{CH}_3)$ is not very dependent on the choice of $\tau_{\text{int}}$, but it is sensitive to changes in $\tau_c$. The value of $10^{-8}$ sec chosen for the rotational correlation time, $\tau_c$, of the protein can be considered a lower limit since the rotational correlation times of BSA have been found to be higher up to one order of magnitude (21). Higher values of $\tau_c$ lead to smaller values of $T_{2I} (\text{CH}_3)$ so that $\tau_{IS} > 0.015$ and consequently at $30^\circ C$

$$30 < k_{-1} < 80 \text{ sec}^{-1}$$

where the experimental errors (table 3) have been taken into account. It should be noted that within these limits $\tau_{IS}$ is of the same order of magnitude as $T_{2I} (\phi-H)$ and smaller than $T_{2I} (\text{CH}_3)$. This is in accordance with the temperature dependence of the linebroadening parameters $X_I (\text{CH}_3)$ and $X_I (\phi-H)$. It is well known that the temperature coefficients of $\tau^{-1}$ and $T_{2I}^{-1}$ have opposite signs, i.e. $\tau^{-1}$ has a positive temperature coefficient, while $T_{2I}^{-1}$ has a negative temperature coefficient (7). Since $X_I$ is found to increase with increasing temperature this indicates the importance of $\tau_{IS}$ in the r.h.s. of equation 10 in agreement with the findings above.

The non-specific acetrizoate albumin complex (class II) shows a different picture. The $X_{II}$ values have negative temperature coefficients. Moreover, definite changes of the resonance positions, $\delta$, are observed upon binding of acetrizoate to albumin. The linebroadening parameters $X_{II}$ and the chemical shift parameters $\Delta_{II}$ of both the methyl and the phenyl protons and their temperature dependence can be described by eqs. 14 and 15 and their temperature derivatives. These form a set of eight equations with eight unknowns which can be solved using the results in table 3. It turned out that these parameters could only be slightly varied within their error limits to give
physically acceptable solutions for $\tau_{II}$, $T_{2II}$ and $\Delta \omega_{II}$ for both the methyl and the phenyl protons. Subsequently the values obtained for the latter parameters were substituted in the more general Swift and Connick equations 7 and 9 and varied in such a way that the differences between the calculated and observed values of $X_{II}$ and $\Delta_{II}$ became minimal. This resulted in the following lifetimes of the acetrizoate albumin complexes (class II):

- at 4°C $\tau_{II} = 2 - 2.5$ msec
- at 30°C $\tau_{II} = 0.4 - 0.6$ msec

These results imply that there is a relatively large exchange contribution to the linebroadening parameter $X_{II}$. However, as would be expected from the temperature dependence of the linebroadening parameters, the $T_2$ parameters do dominate; i.e. at 30°C

- $T_{2II} (\phi-H) = 17 - 23$ msec
- $T_{2II} (\text{CH}_3) = 16 - 18$ msec

At 4°C these values are about halved. The $\Delta \omega$ values are indeed found to be of the same order of the $T_{2II}^{-1}$ values, in accordance with the assumptions leading to eqs. 14 and 15 i.e.

- $\Delta \omega (\phi-H) = 220$ rad/sec
- $\Delta \omega (\text{CH}_3) = 80$ rad/sec
DISCUSSION

A. THE MODE OF BINDING OF ACETRIZOATE AT TYPE II BINDING SITES

From the results above we find for the spin-spin relaxation rates of the methyl and the phenyl protons:

\[
T_{2II}^{-1} (\delta-H) = 50 \pm 10 \text{ sec}^{-1} \quad \text{at } 30^\circ C
\]

\[
T_{2II}^{-1} (\text{CH}_3) = 59 \pm 4 \text{ sec}^{-1}
\]

The spin-spin relaxation time is determined by the correlation time, \( \tau_c \), which is characteristic of the tumbling time of the molecule or of an individual molecular group, and by the distances between the nucleus considered and magnetic moment bearing nuclei in the surroundings. Thus the \( T_2 \) values of different groups of acetrizoate may yield information about their mobility with respect to the BSA molecule.

In the bound state the spin-spin relaxation time of the aromatic proton will be determined predominantly by protein nuclei at an unknown distance. Thus little can be said about the mobility of the phenyl group from \( T_2 \) values alone. However, the ratio of the spin lattice relaxation time, \( T_1 \), over the spin-spin relaxation time, \( T_2 \), is only a function of \( \tau_c \) and in a temperature and frequency dependent study of the binding of sulfonamide to carbonic anhydrase Lanir and Navon (23) have shown that these ratios yield reliable \( \tau_c \) values. For this reason spin lattice relaxation rates, \( T_1^{-1} \), have been determined under the same conditions the \( T_2 \) experiments were carried out. For \( T_1^{-1} \) a formula analogous to equation 6 can be derived. \( T_1 \) measurements at various BSA and acetrizoate concentrations yielded a value of

\[
T_{1II}^{-1} (\delta-H) = 10 \text{ sec}^{-1} \quad \text{at } 30^\circ C.
\]

From the ratio \( T_{1II}/T_{2II} = 5 \) we obtain \( \tau_c = 3 \times 10^{-9} \text{ sec} \quad \text{at } 30^\circ C. \)

\( T_1 \) measurements of the methyl group exhibited only slight changes with respect to the non-bound situation except for the disappearance of the non-exponential behavior (22). As a result the \( T_{1II} (\text{CH}_3) \) could not be established with sufficient accuracy and the correlation time was directly calculated from the \( T_{2II} (\text{CH}_3) \) yielding a value of \( 3 \times 10^{-9} \text{ sec}. \) Again Fig. 1 of the
paper of Werbelow and Marshall (20) was used, while assuming that the rotation of the methylgroup around its axis is not restricted. The results are listed in table 4 together with the values for nonbonded acetrizoate and for albumin. Examination of table 4 shows that although the mobility of the phenylgroup of acetrizoate is slowed down as a result of binding to the type II binding sites, it still has a considerable amount of motional freedom with respect to the BSA molecule. The $\tau_c$ value obtained for the methylgroup is equal to the value obtained for the phenylgroup, which indicates that the phenylgroup and the methylgroup have about equal mobility on the surface of the protein, except for the rotation of the methylgroup around its axis.

B. THE INFLUENCE OF THE BINDING OF ACETRIZOATE TO ALBUMIN ON THE RENAL EXCRETION

At clinical doses of 200 mg of acetrizoate per kg body weight, plasma levels occur up to 1 mM (10). At this concentration about 70% is bound to bovine albumin (and probably somewhat less to human albumin (12)) of which about three quarters is bound to the first specific binding site. Above the dissociation rate of type I complexes was found to be in the region $30 < k_1^- < 80 \text{ sec}^{-1}$, which corresponds to a lifetime of the albumin acetrizoate complex between 0.03 and 0.012 sec. This is at least an order of magnitude less than the cortical transit time which may vary from .3 to 3 sec (24, 25). Thus in a first approximation little influence of the dissociation rate on the active tubular excretion of acetrizoate is to be expected. However, such a conclusion should be regarded with some care. Although the dissociation rate is high with respect to the reciprocal transit time, the high affinity constant of the acetrizoate albumin complex results in a very high association rate constant which might compete with the excretion rate. Model calculations (26) show that in the region where the albumin complex lifetime equals the transit time the system is most sensitive to changes in dissociation rate and that irrespective of the dissociation rate, protein binding decreases the amount of actively excreted drugs as long as the maximum transport capacity has not been reached.
Table 4
Correlation times at 30°C

<table>
<thead>
<tr>
<th></th>
<th>correlation time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>sec⁻¹</td>
</tr>
<tr>
<td><strong>Albumin</strong> a)</td>
<td>(2 - 20) x 10⁻⁸</td>
</tr>
<tr>
<td>Acetrizoate CH₃ bound to</td>
<td>3 x 10⁻⁹</td>
</tr>
<tr>
<td>type II ϕ-H binding sites</td>
<td>3 x 10⁻⁹</td>
</tr>
<tr>
<td>Acetrizoate CH₃ free in</td>
<td>8 x 10⁻¹²</td>
</tr>
<tr>
<td>solution b) ϕ-H</td>
<td>2.4 x 10⁻¹⁰</td>
</tr>
</tbody>
</table>

a) D. Wallach (21)
b) J.F. Rodrigues de Miranda and C.W. Hilbers (22)

CONCLUSIONS

It has been shown, by a combination of nmr and equilibrium dialysis experiments, that the rather complicated binding of acetrizoate to albumin can be unraveled. Binding constants for two different classes of binding sites have been obtained.

The lifetime of the specific albumin acetrizoate complex (class I) is in the region of 0.012 to 0.03 sec. This value is much less than the cortical transit time; further decrease of the lifetime of the complex will have little influence on the renal active excretion rate. Comparison of the kinetic parameters obtained for the two classes of binding sites shows that the difference in the corresponding binding constants is mainly caused by the difference in the association rate constants i.e. the decrease in the binding constant of 4.6 x 10⁵ M⁻¹ for type I binding sites to 120 M⁻¹ for type II binding sites mainly comes from the decrease of k⁺₁ = 2.5 x 10⁷ sec⁻¹ M⁻¹ to k⁺₁₁ = 2 x 10³ sec⁻¹ M⁻¹ rather than from an increase in the dissociation rate constants. Such a
phenomenon has been observed earlier for the sulfonamide binding to carbonic anhydrase (27). Hence also in the present study the association between protein and substrate cannot be described by a diffusion-limited complex formation.

The mobility of the acetrizoate molecule when bound to the second class of binding sites could be estimated. It turned out that in this case the correlation time of the acetrizoate molecule was an order of magnitude less than the correlation time of the protein, indicating an appreciable amount of motional freedom with respect to the protein.

The potential possibilities which nmr offers for studying the molecular dynamics of substrate on protein surfaces has led to a number of linebroadening studies of pharmacologically highly interesting systems. In these investigations linebroadenings have often been interpreted to yield values of $T_e^{-1}$ (bound) of the order of $10^{-4}$ sec$^{-1}$ which correspond to linewidths of the substrate resonances of the order of $10^3$ Hz (28, 29). These values are improbably large, especially when one realizes that these linewidths exceed the total spectral width of most proteins hitherto observed at 100 MHz. Moreover, the individual resonances of protein residues for instance of hemoglobin are of the order of 100 Hz (7) again indicating the unlikely high value of $T_e^{-1}$ (bound) obtained in some of these binding studies. It should therefore be stressed that before interpreting linewidth alterations, the exchange limit has to be established, moreover, it should be checked whether linebroadening is a result from binding to only one, or one class, of binding site(s) in the concentration region studied in the nmr experiments.

ACKNOWLEDGEMENT

The experimental assistance of A.J.G.M. Henderix and J.W.M. van Kessel is gratefully acknowledged.
REFERENCES

THE KINETICS OF THE BINDING OF ACETRIZOATE TO ALBUMIN
IN RELATION TO ITS RENAL CLEARANCE

J.F. Rodrigues de Miranda*, M.A. van 't Hof+ and C.W. Hilbers*

University of Nijmegen
Nijmegen, The Netherlands.

* Department of Pharmacology
+ Department of Statistical Consultation
◆ Department of Biophysical Chemistry
The renal and hepatic clearance of drugs, which bind to plasma proteins, are concentration dependent, resulting in non-linear kinetics of elimination (1). In the kidney the protein bound fraction is not susceptible to ultra filtration, whereas with regard to active tubular excretion, protein binding may be interfering. Little is known about the influence of the dissociation rate of the protein-drug complex on the effectiveness of the active transport (2).

In the present study the dissociation rate of the contrast medium acetrizoate (Triognost\textsuperscript{R}) is related to the active tubular excretion rate (3, 4). To this end the dissociation rate constant of the acetrizoate-albumin complex was deduced from nmr experiments.

\begin{center}
\includegraphics[width=0.5\textwidth]{acetrizoate.png}
\end{center}

Acetrizoate

Binding of substrate molecules to macromolecules may cause an increase in the linewidths of the nmr signals of the substrate molecules (5). If an excess of substrate is used the line broadening is proportional to the fraction, \( f_b \), of substrate bound to the macromolecule:

\[ \text{line broadening} = f_b \cdot X \text{ sec}^{-1} \]  

(1)

The line broadening parameter \( X \) is a complicated function of the relaxation rate \( T_{2b}^{-1} \), the exchange rate \( \tau^{-1} \) and the chemical shift differences between the resonances of the substrate bound to the macromolecule and free in solution (6). \( T_{2b}^{-1} \) is the relaxation rate of the substrate molecule bound to the protein and \( \tau^{-1} \) equals the dissociation rate \( k^- \) of the protein-substrate complex. In absence of these chemical shift differences eq. 1 can be simplified to:

\[ \text{line broadening} = f_b \cdot (\tau + T_{2b})^{-1} \]  

(2)

An example of the line broadening observed for the acetrizoate resonances due to binding to albumin is shown in Fig. 1.

The lower half of the figure displays the spectrum of a 6 x 10\textsuperscript{-3} M acetrizoate solution without albumin. The upperhalf of the figure was recorded in the presence of 2.4 x 10\textsuperscript{-5} M albumin. The intense signal at about 430 Hz is due to residual HDO. The resonances of interest at 58, 160, 260 and 780 Hz respectively, recorded on an expanded scale.
and at higher amplification factor, were inserted in the figure at their corresponding frequencies.

Fig. 1. nmr spectra of acetrizoate in $D_2O$-phosphate buffer, pH 7.4. Lower spectrum without, upper spectrum with albumin.

Proceeding from left to right they represent the $\phi$-H of acetrizoate, the methylgroups of tetramethylammoniumchloride, the methylgroup of acetrizoate and the methylgroups of t-butylalcohol. The methyl resonances of the tetramethylammoniumchloride and t-butylalcohol were used as a check on the field homogeneity. Addition of albumin clearly shows a line broadening of the acetrizoate resonances while the reference signals remain virtually unchanged.

Since the temperature coefficients of $T_2$ and $\tau$ in eq. 2 are different in sign, the temperature dependence of the line broadening can be employed to establish whether the relaxation rate or the exchange rate dominates in the line broadening parameter. The line broadening caused by the binding of acetrizoate to the specific binding site on
albumin displays a positive temperature coefficient indicative of a slow exchange situation. This, together with the inequality of the line broadening parameters of the methyl and aromatic protons sets the following limits to the dissociation rate constant (7):

$$80 > k^- > 30 \text{ sec}^{-1}$$

This dissociation rate constant corresponds to a mean lifetime of the acetrizoate-albumin complex of 0.03 - 0.012 sec which is one order of magnitude smaller than the mean cortical transit time of 0.3 - 3 sec (89). Hence little influence of the dissociation rate on the effectiveness of the active excretion is to be expected (2). Such a conclusion should, however, be regarded with some care. To obtain a more quantitative view of the influence of protein binding and of the dissociation rate of the drug-protein complex on the active excretion rate, differential equations were designed based on simple Michaelis-Menten kinetics and a non-equilibrium situation for the protein-pharmaccon interaction. By a numerical procedure excretion curves were calculated; for an example see Fig. 2. Plotted are the relative tubular active excretion $E/T_m$, with $E$ being the tubular active excretion rate and $T_m$ the tubular maximum rate, versus the total concentration of substrate in the plasma, $C$, in mM. The excretion curves have two boundaries, indicated by the dashed lines. The horizontal asymptote ($E = T_m$) reflects the capacity limit of the excretion system, the sloping one the flow limit of the system ($E = V \times C$) with $V$ being the plasma flow through the system. For the situation indicated i.e. a dissociation equilibrium constant, $D$, of $5 \times 10^{-5}$ M for the albumin-substrate complex, $5 \times 10^{-5}$ M for the carrier-substrate complex, $K$, and an albumin concentration, $A$, of $5 \times 10^{-4}$ M, excretion curves were calculated for three different dissociation rates ($\beta = k^-/T$).

1. No dissociation of the protein-acetrizoate complex occurs: $\beta = 0$.
2. The lifetime of the albumin complex is very much shorter than the transit time $T$: $\beta \rightarrow \infty$
3. The lifetime of the complex equals the transit time through the pars recta: $\beta = 1$.

For reasons of comparison a fourth curve, $(A = 0)$, representing the situation with no protein binding, is also given. From these curves it should be noted that even in case the dissociation rate constant is very high, $10^6 \text{ sec}^{-1}$ say, the protein binding still diminishes the
effectiveness of the active excretion.

Fig. 2. Plot of the active drug excretion as a function of the total drug concentration in the plasma for different dissociation rates of the drug-albumin complex.

\[ V = 60 \text{ mL/min}; \ T = 30 \mu\text{mol/min}; \ K = 5 \times 10^{-5} \text{ M}; \ D = 5 \times 10^{-5} \text{ M}; \]
\[ \lambda = 5 \times 10^{-1} \text{ M}. \]
Symbols are defined in the text.

In conclusion it can be said that

i) Irrespective of the dissociation rate, protein binding decreases the amount of drug actively excreted as long as the maximum transport capacity is not reached.

ii) In the region where the albumin complex lifetime equals the transit time the system is very sensitive to changes in \( \beta \).

More extended calculations will be presented elsewhere.
REFERENCES


SECTION III
SPECIFICITY IN DIASTEREOMERIC INTERACTIONS
STEREOSELECTIVITY AND AFFINITY IN MOLECULAR PHARMACOLOGY.

1. THE CORRELATION OF STEREOSELECTIVITY AND ACTIVITY

P.A. Lehmann F.†, J.F. Rodrigues de Miranda and E.J. Ariëns
Pharmacology Institute, University of Nijmegen,
Nijmegen, The Netherlands.

† On Sabbatical Leave from the Departamento de Farmacología y Toxicología, Centro de Investigación y Estudios Avanzados, Instituto Politécnico Nacional, A.P. 14-740, México, D.F., México.
When the biological activities of stereoisomers (enantiomeric and diastereomeric, such as epimeric or geometric) are compared, it is sometimes found that only one member of each pair is active, often that both members are equally active. Very frequently though, both members exhibit the same type of activity but to a different degree. Several thousand such cases have been recorded (see references 1-79 and other literature summarized in 80-85), most of them for structurally specific drug action and certain types of enzymatic action. The differences in activity for the members of any one pair (most conveniently expressed as the ratio of their activities or potencies) vary enormously, viz. from ca. 1 up to nearly $10^6$. In spite of extended efforts (80-85), it has so far not been possible to explain these ratios; every biological system, sometimes every individual pair, seems to constitute a casus sui generis.

Actually this failure is partly understandable in view of the complexity of receptor-drug and enzyme-substrate interactions (differences in intrinsic activity, conformational mobility, simultaneous interaction at more than one point, multiple concurrent forms of interaction, dualism of action, etc.) and of the experimental difficulties which may intervene (faulty estimation of stereoisomeric purity, selective distribution, accuracy of potency estimates over large spans, limited availability of appropriate test cases, enzyme purity, complex formation between stereoisomers, etc.).

Two studies (86, 87) are available, however, in which attention is drawn to the fact that the ratio of potencies increases with an increase in the potency of the racemate or of the more potent isomer. The seemingly accidental nature of these correlations, as well as the lack of a theoretical basis for them, has resulted in their neglect by most workers in the fields concerned with this phenomenon. If such correlations are in fact not accidental, their analysis should be useful in exploring a fundamental aspect of S.A.R. general to all drug action.

The present work summarizes the results of a literature search aimed at establishing the frequency with which a correlation appears either between the potencies of the isomers or directly between the ratio of their potencies and the potency of one of them.
ANALYSIS OF EUDISMIC-AFFINITY CORRELATIONS

Discussion of this topic is greatly facilitated by the introduction of some new terms defined as follows. In any pair of isomers for which a given biological system shows stereoselectivity, the more active one will be termed the eutomer (Eu) and the less active one the distomer (Dis) regardless of their absolute configuration. A homologous series of pairs will then consist of a eutomeric series and a distomeric series. These are necessary designations because they classify a pair on the basis of complex stability. This, which is a consequence of the geometry of the active site on the biomacromolecule, is determinant, rather than the geometry of the isolated drug which may be irrelevant. For any given pair the ratio of their potencies (activities, affinities, efficiencies as substrates, etc.) will be termed the eudismic ratio and its logarithm, the eudismic index (E.I.), in analogy to the stereoselectivity index used by other authors (e.g. 8). Finally, in a homologous series, the rate of change of E.I. for a given increase in log potency of the eutomer (log Eu, which usually may be taken as a measure of its affinity), will be called the eudismic-affinity quotient (E.A.Q.). The meaning of these terms should become clear from the illustrative example given in Fig. 1. Consider a set of three isomeric pairs whose relative potencies are as shown in Fig. 1a. Logarithmic scales are used not only for compactness but also because a scale is obtained which is independent of the units employed in the potency estimates.

* The use of these terms, derived from ευ = good, δυζ = bad and μεροζ = part, is suggested in analogy to the previously proposed designations of eutopic (well-fitting) and distopic (ill-fitting) complexes (88).
After conversion of the potency estimates to logarithmic units, any correlation between the eutomeric and distomeric series will become evident in either of two ways. In Fig. 1b both have been plotted against a common scale. Obviously log Eu against log Eu gives a straight line of slope 1; but a line dropped from any point on it to the point corresponding to the distomer represents the drop in activity i.e., its E.I.

<table>
<thead>
<tr>
<th>Pair No</th>
<th>Isomer No</th>
<th>Relative Potency</th>
<th>log Relative Potency</th>
<th>Eudismic Index (E.I.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Eu 1</td>
<td>100</td>
<td>2.00</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>Dis 1</td>
<td>25</td>
<td>1.40</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Eu 2</td>
<td>1,000</td>
<td>3.00</td>
<td>1.15</td>
</tr>
<tr>
<td></td>
<td>Dis 2</td>
<td>70</td>
<td>1.85</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Eu 3</td>
<td>10,000</td>
<td>4.00</td>
<td>1.70</td>
</tr>
<tr>
<td></td>
<td>Dis 3</td>
<td>200</td>
<td>2.30</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Eudismic-affinity correlation plots, illustrative example.  
(a) Table of potencies for a three-pair set.  
(b) Separate plots of log Eu and log Dis each vs log Eu; note that the vertical distance between any pair of points corresponds to the potency drop from Eu to Dis for that pair.  
(c) Eudismic-affinity correlation plot. The equation for the line shown is E.I. = a + b log Eu, where b = tan $\theta$ = E.A.Q. and log Eu$_{log}$ = $-a/b$. These terms are related to the line equation for the distomeric series in b. through $b' = 1 - b$ and $a' = -a$ for the equation log Dis = $a' + b' log Eu$.  

91
The plot shown in Fig. 1c is an even more compact and convenient representation in which the E.I. has been plotted directly against log Eu for each pair. The slope of the line obtained then corresponds to the change in E.I. per unit change in log Eu, that is the E.A.Q. for this system. The intercept of this line with the abscissa will be designated log Eu₀.

This type of plot is similar to that used previously by Pfeiffer (86) and Ariëns and Simonis (87), but differs in that increases in potency are plotted to the right, and that all points appear in the upper quadrants, which follows from the definition of Eu and Dis. From this it may be seen that although the E.I. is always positive, the E.A.Q. may be positive, negative or zero. Each case corresponds to a different kind of relationship which our data show to exist.

This type of plot is not only a compact way of summarizing eudismic data, but also displays it in such a way that both visual and mathematical analyses of any existing correlations become simple. It is used in the examples which follow and is the basis of the data presented in Tables 1 and 2.

EXAMPLES OF SOME EUDISMIC-AFFINITY CORRELATIONS

Before presenting the results of the survey, it is useful to introduce the subject by discussing some examples, drawn from the most diverse possible fields (pharmacology, enzymology, plant physiology), representative of different types of action (chemical reactivity, agonism, antagonism, etc.), and of different classes of stereoisomerism (enantiomeric, epimeric and geometric).

In addition, some have been selected because they show clearly certain features of eudismic-affinity correlations which we have found to be of general occurrence.

A figure is given for each example which contains the following information: (a) Type of biological activity studied. (b) Structures of the stereoisomeric pairs, coded to their position on the plots; an asterisk indicates that a given eutomer has an absolute configuration opposite to that of the eutomeric series as a whole, and a vertical
arrow above a point indicates that only a minimum estimate of the E.I. was available. No error estimates are to be inferred from the size of the circles about the points. (c) The lines shown are least-squares' estimates through the pertinent points, and the slopes indicated correspond to their E.A.Q. (d) Each eudismic-affinity correlation (E.A.C.) is numbered as it appears in Tables 1 and 2, where further data concerning it can be found.

The activity correlated may be pharmacological activity ($pA_2$, $pD_2$, dose, relative potency, etc.), or an enzymological parameter ($k_{cat}$, $K_M$, $K_I$, $k_{cat}/K_M$, turnover, etc.). The parameter is taken in such a way that its dimension is $M^{-1}$ or proportional to it.

**ISOPROPYLPHOSPHOTHIONATES**

The inactivation of cholinesterases by isopropylphosphothionates (Fig. 2) involves the alkylation of two different enzymes, presumably preceded by the formation of an activated complex:

$$E + RX \rightarrow [E.RX] \rightarrow \text{products}$$

![Diagram of ISOPROPYLPHOSPHOTHIONATES](image)

**Fig. 2. Inactivation of cholinesterase by isopropylphosphothionates.**

- **a.** Acetylcholinesterase (E.A.C. 101)
- **b.** Butyrylcholinesterase (E.A.C. 103)
The following points may be noted:
1. The nine alkylating phosphothionates vary tremendously in their reactivity towards each enzyme since a 5-decade span of activity is covered. In general both enzymes show a similar reactivity toward any given phosphothionate, i.e. circles and squares fall approximately at the same point on the log Eu scale.
2. The acetylcholinesterase (a) displays striking behavior: as the reactivity of the eutomer increases, the E.I. increases also in a regular fashion. The correlation between the two variables is highly significant and 91% of the variance is explained by linear regression. The slope of the correlation line is an excellent measure of the stereoselectivity of this enzyme toward this homologous series of inactivators.
3. The butyrylcholinesterase (b) behaves in a completely different way: throughout the 4-decade span of reactivity the eutomer is not significantly more reactive than the distomer. That is, for it the E.I. is constant and not significantly different from zero, and E.A.Q. = 0. This indicates (cf. Ariëns and Simonis, 87) that the chirality of the alkylating agents plays no role whatsoever in the alkylation of this particular enzyme.

DIPHENHYDRAMINE DERIVATIVES

The second example (Fig. 3) is concerned with the antihistaminic and anticholinergic activities of some diphenhydramine derivatives. These correlations are typical of a large number of cases encountered for pharmacological antagonists. Note that:
1. Relatively high affinities are involved for both systems.
2. The eutomeric series follows a different sequence in each system for the same homologous series of compounds, indicating that the receptors involved are quite different.
3. Each set includes one pair in which the eutomer is of opposite absolute configuration to that of the rest of the series (points with asterisks). These can nevertheless be correlated; i.e. affinity, not absolute configuration, is of primary importance. With a few exceptions, however, such members of a series usually have low affinities.
Fig. 3. Activities of diphenhydramine derivatives.

a. Antihistaminic (E.A.C. \( \geq 14 \))
b. Anticholinergic (E.A.C. \( \geq 12 \))

**MUSCARINIC 1,3-DIOXOLANES**

Some activity correlations of muscarinic 1,3-dioxolanes are shown in the next example (Fig. 4). In this figure we examine the interaction of one homologous series of substituted dioxolanes with two different "receptors": the muscarinic receptor (a and b) and acetylcholinesterase (c).

The lower members of the series (1-6) are agonists which mimic AcCh at the muscarinic receptor of guinea-pig ileum smooth muscle. The line indicated in (a) was obtained from the four points reported in one study (Chang and Triggle 1973, 3). The points whose numbers are in parentheses are from a separate study (Belleau and Lavoie 1968, 18) and were not included in the correlation but are not out of line. Higher homologs (8-10) are antagonists (b) at the same receptor.

Two important points are apparent from those correlations:

1. The E.A.Q. is similar for both agonists and antagonists.
2. The log \( \text{Eu} \) value is much higher for antagonists than agonists.

Note that pairs 5 and 6 in (a) and (c), and 9 and 10 in (c) can also
be correlated, although two, chiral centers are present. A further interesting point is that for agonists the E.I. increases as the size of the R' group diminishes.

![Graph showing activities of muscarinic 1,3-dioxolanes](image)

**Fig. 4. Activities of muscarinic 1,3-dioxolanes.**

- a. Muscarinic agonists (E.A.C. 1)
- b. Muscarinic antagonists (E.A.C. 5)
- c. Acetylcholinesterase inhibition (E.A.C. 135)

*(ABMC is acetyl-β-methylcholine)*

**ACETYLCHOLINESTERASE**

The work of Belleau and Lavoie on the interaction of three members (4-6) as competitive inhibitors of acetylcholinesterase, enables us to further extend this comparison. Plot (c) shows us that where *in vitro* affinity constants are compared, a very high correlation is observed. Note that the stereoselectivity of acetylcholinesterase is higher than that of the muscarinic receptor.
The excellent correlation obtained in the comparison shown in Fig. 5 is truly remarkable for the following reasons:

1. Changes at two chiral centers are involved.
2. Changes at two different positions are included.
3. The results are from an in vivo assay on a whole animal involving the antagonism (presumably competitive interaction) of two drugs on the analgetic receptor in the CNS.

Fig. 5. Meperidine-analgesic antagonism by bornomorphan derivatives. (E.A.C. ? ?).

ANTIHISTAMINES

In contrast to the previous examples, all of which involved true enantiomeric pairs resulting from the presence in the molecules of a chiral P or C atom, the next example (Fig. 6) involves pairs of geometric isomers (cis/trans) about a double bond, that is, the comparison is made between the E and Z isomers of a given unsymmetrical
olefin instead of between the R and S form of a chiral compound.

![Chemical structures and graph]

**Fig. 6. Antihistaminic activity of aryIpropanes and -butenes. (E.A.C. % 6).**

**SUBTILISIN**

Although a complex quantity, the ratio \( k/K_M \) is accepted by enzymologists as a good measure of the "efficiency" of a substrate in typical enzymatic reactions.

The following points are noteworthy in regard to Fig. 7:

1. Log Eu values range over 3.5 decades and E.I. values over 4 decades.
2. At least three analogs of the "wrong" absolute configuration show high activities, but can be correlated well.
3. The E.I. for 8 and 10 are minimum estimates, but judging from the overall correlation, should not be too far off (not included in correlation).
4. Separate correlations of \( k \) (E.A.C. % 105) and \( K_M^{-1} \) (E.A.C. % 122) are also significant, but have different E.A.Q.'s.
Fig. 7. Subtilisin-BPN' hydrolysis of N-acetyl phenylalanine methyl ester and structural analogs. 
(Ø = phenyl; Nyl = naphthyl; In = indanyl)

AUXINS

The availability of no less than 32 pairs of closely related auxin analogs (Fig. 8) provides an outstanding test system which strikingly confirms earlier observations and allows some new ones.

1. It is apparent that the points fall into two groups: those clustered around line (a) and those about line (b). Each of these corresponds to a different structural type: in the first a link is present between the aryl moiety and the chiral center, whereas in the second there is none.

2. The analogs grouped together in set (a) include a large variety of different substituents and substitutions. If the criterion for homology is made more stringent by considering a subset of analogs in which only one substituent is varied, a better correlation may be expected. This prediction is strikingly verified by subset (a') (darkened circles)
which singles out six α-(2-naphthoxy)propionic acids. The coefficient of determination for regression to the line indicated for this subset (E.A.Q. = 1.07) is 0.991.

3. The correlation shown for the α-arylpropionic acids (b) is interesting in two respects:
   firstly, it shows a negative E.A.Q., that is for it stereoselectivity decreases with an increase in affinity. Secondly, the intercept of this correlation line on the abscissa is exactly at the point corresponding to an achiral homolog of the series, namely indoleacetic acid, the natural plant hormone.

![Graph showing inhibition of flax-root growth by auxin-like aryloxyarboxylic acids](image-url)

**Fig. 8.** Inhibition of flax-root growth by auxin-like aryloxyarboxylic acids.
   a. Aryloxyarboxylic acids (E.A.C. ¶ 8) ○
   a'. β-naphthylaryloxyarboxylic acids ●
   b. Arylpropionic acids (E.A.C. ¶ 11) ●
   The corresponding structures may be found in the original references (7ab), I.A.A. = indoleacetic acid ●
In this phase it should be realized that as far as the ratio of potency of two isomers is concerned, a slight contamination of the eutomer (1 to 2% of the less active isomer) does not influence the ratio essentially.

If the poorly active or inactive isomer (distomer), however, is contaminated with 1 to 2% of the eutomer, the ratio (ER) may be drastically changed. As a rule therefore the ratio found is the lower limit. This also makes it understandable that ratios over 200 are exceptional. A contamination will also contribute to a scatter of the experimental points and implies a downward deviation of the curve at higher log Eu values.

From the above mentioned examples it should now be obvious that stereoselectivity does not occur randomly but may be correlated with log Eu.

The diversity of circumstances under which correlations are apparent points to the existence of a simple principle, fundamental to them.

SURVEY OF PUBLISHED STEREoseLECTIVITY DATA

The examples presented have shown that at least for them the eudismic index can be correlated with log Eu, although the relation between them is not necessarily only an increasing one. To find out if these are in some way exceptional cases or whether they are in fact just especially obvious cases of a generally occurring relationship, we have carried out a survey of published data on this topic covering a total of 700 pairs. Although not exhaustive, the data included are representative of a variety of systems, isomerism, etc., and include a large portion of the more important work undertaken specifically on the problem of stereoselectivity. For reasons of saving space only the data covering a log Eu span larger than 1 are included in tables 1 & 2.

The basic criterion used for inclusion of data was the reporting separately of quantitative activity estimates for each member of two or more isomeric pairs on the same biological or other chiral "receptor"
DESCRIPTION OF THE TABLES

The data gathered have been separated into two groups: In group I (part of which is given in table 1) are included those sets in which an in vivo system was employed, such as tissue cultures, isolated organs, whole animals, and microbial colonies, i.e. pharmacological systems. In group 2 (part of which is given in table 2) we have placed the sets referring to in vitro test systems such as pure enzymes, or containing artificial chiral components (silica with chiral "foot-prints", chiral metal chelate complexes, etc.) corresponding to such biomacromolecules. The sets are ranked in order (decreasing) of the spans covered by the eutomeric series.

For each set is given:
1. A set correlation number (E.A.C. %).
2. A concise description of the type of activity involved, the class of compounds studied, and the chiral center "epimerized" if more than one is present. The type of isomerism involved (O, E or G) is indicated between brackets together with the number of pairs included.
   
   O = enantiomeric (at carbon or phosphorus)
   E = epimeric (linear or cyclic)
   G = geometric (in the sense of cis/trans)

Achiral lower homologs (e.g. glycine among amino acids) have not been included.
3. The parameter correlated.
4. The log Eu span covered.
5. The E.A.Q. value and the significance of a given correlation.
   Two levels of significance are used: * P < 5.0, + = 5.0 < P < 10.0, where P is the probability (in %) that the absolute t-statistic is greater than the determined t-value on the assumption that there is no correlation.
   
   For sets with E.A.Q. close to zero (recognizable by the occurrence of an entry in the next to last column), the significance of the correlation was established by a direct comparison of log Eu and log Dis values, i.e. corresponding to plots such as the one in Fig. 1b.
6. The coefficient of determination \( r^2 \); when multiplied by 100 it gives the percentage of variance explained by the linear regression to the calculated least-squares' line.
7. For those cases with E.A.Q. between -0.3 and +0.3 and a P value of
> 10 for the E.A.Q. correlation, is given the average E.I. for such
series. Its S.D. is included to permit establishing whether it is sig­
nificantly different from zero.

8. The reference(s) from which the data were obtained.

ABBREVIATIONS USED IN THE TABLES

E.A.C. ℓ = eudismic-affinity correlation set number; isom. = isomer;
0 = enantiomeric (optical); E = epimeric; G = geometric; E.A.Q. = eudis­
mic-affinity quotient; Ave. E.I. (S.D.) = average eudismic index and
standard deviation for sets with E.A.Q. not significantly different
from zero; Ref. = reference.
A.a. = amino acid; AcCh = acetylcholine; act. = activity; alt. = al­
tered; antichol. = anticholinergic; D = dose; e.p. = electro shock
protection; est. = esterase; g.p.i. = guinea pig ileum; hydrol. = hydro­
ysis; inact. = inactivation; inh. = inhibition; k_d = dissociation
constant; lr = logarithm of the rate; p = negative logarithm;
parasymp. = parasympathomimetic; r.b.c. = red blood corpuscle;
r. = renal; rel. = relative; r.i. = rat intestine; r.p. = relative po­
tency; synth. = synthetase.
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<th>E.A.C.</th>
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for pharmacological systems

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105
Table 1, continued

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<td>Hexobarbital potentiation by tropinic phenothiazines ([E;3])</td>
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In order to save space the E.A.C.'s 40 - 57 with more than two pairs in their sets (Ref. 37 - 49) are excluded from this table; they are
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<td>0.49</td>
<td></td>
<td>13</td>
</tr>
<tr>
<td>pA_2</td>
<td>1.04</td>
<td>0.13*</td>
<td>0.73</td>
<td>0.3(.2)</td>
<td>8</td>
</tr>
<tr>
<td>pED_{30}</td>
<td>1.00</td>
<td>0.06</td>
<td>0.95</td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>pED_{50}</td>
<td>1.00</td>
<td>0.09*</td>
<td>0.93</td>
<td>0.2(.1)</td>
<td>26</td>
</tr>
<tr>
<td>pC_{S20}</td>
<td>1.00</td>
<td>0.86</td>
<td>0.59</td>
<td></td>
<td>7b</td>
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</table>

in their sets (Ref. 27 - 36) and the E.A.C.'s 58 - 88 with two pairs however included in table 3.
<table>
<thead>
<tr>
<th>E.A.C.</th>
<th>Description of activity, enzyme and substrate series</th>
</tr>
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<tbody>
<tr>
<td>101</td>
<td>Inact. of r.b.c. AcCh-est. by iPr-phosphothionates [0;9]</td>
</tr>
<tr>
<td>102</td>
<td>α-CT hydrol. of methyl esters [0;14]</td>
</tr>
<tr>
<td>103</td>
<td>Inact. of butyrylcholinester. by iPr-phosphothionates [0;9]</td>
</tr>
<tr>
<td>104</td>
<td>Inact. of beet-brain AcCh-est. by iPr-phosphothionates [0;6]</td>
</tr>
<tr>
<td>105</td>
<td>Subtilisin BPN' hydrol. of methyl esters [0;12]</td>
</tr>
<tr>
<td>106</td>
<td>Carboxypeptidase hydrol of L-Phe amides [E;3]</td>
</tr>
<tr>
<td>107</td>
<td>α-CT hydrol. of Z-a.a. PNP esters [0;7]</td>
</tr>
<tr>
<td>108</td>
<td>Carboxypeptidase-A hydrol. of [3]-alt. Ala-peptides [E;8]</td>
</tr>
<tr>
<td>109</td>
<td>α-CT hydrol. of N-acylalanine methyl esters [0;10]</td>
</tr>
<tr>
<td>110</td>
<td>Inact. of aliesterase by iPr-phosphothionates [0;6]</td>
</tr>
<tr>
<td>111</td>
<td>Inact. of r.b.c. AcCh-est. by PNP phosphothionates [0;5]</td>
</tr>
<tr>
<td>112</td>
<td>Inh. of AcCh-est. by piperidinium salts [E;4]</td>
</tr>
<tr>
<td>113</td>
<td>Renal acylase-I hydrol. of α-chloropropionyl amides [E;4]</td>
</tr>
<tr>
<td>114</td>
<td>α-CT hydrol. of methyl esters [0;14]</td>
</tr>
<tr>
<td>115</td>
<td>L-A.a.-oxidase act. (Naja flava) on β-alt. a.a. [E;3]</td>
</tr>
<tr>
<td>116</td>
<td>R. acylase-I hydrol. of [2]-alt. alanyl dipeptides [E;4]</td>
</tr>
<tr>
<td>117</td>
<td>Inact. of α-CT by PNP phosphothionates [0;5]</td>
</tr>
<tr>
<td>118</td>
<td>Hydrol. of [1]-altered glycine dipeptides [0;16]</td>
</tr>
<tr>
<td>119</td>
<td>Glutamine-synth. catalyzed reaction of glutamates [0,E;5]</td>
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<tr>
<td>120</td>
<td>Carboxypeptidase-A hydrol. of [3]-alt. Ala peptides [E;8]</td>
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<tr>
<td>121</td>
<td>α-CT hydrol. of acylalanine methyl esters [0;8]</td>
</tr>
<tr>
<td>122</td>
<td>Subtilisin BPN' hydrol. of methyl esters [0;12]</td>
</tr>
<tr>
<td>123</td>
<td>Inact. of butyrylcholinest. by PNP phosphothionates [0;5]</td>
</tr>
<tr>
<td>124</td>
<td>Lipolytic in vitro act. of adrenergic amines [0;4]</td>
</tr>
</tbody>
</table>
for enzymological systems

<table>
<thead>
<tr>
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<td>lk</td>
<td>4.35</td>
<td>0.60*</td>
<td>0.34</td>
<td></td>
<td>52</td>
</tr>
<tr>
<td>lr</td>
<td>4.18</td>
<td>0.06*</td>
<td>0.97</td>
<td>0.5 (.3)</td>
<td>51</td>
</tr>
<tr>
<td>lr</td>
<td>3.59</td>
<td>0.67*</td>
<td>0.92</td>
<td></td>
<td>51</td>
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<tr>
<td>lk</td>
<td>3.18</td>
<td>1.33*</td>
<td>0.64</td>
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<td>52bc</td>
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<tr>
<td>V_o</td>
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<td>0.87</td>
<td></td>
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<tr>
<td>k_3</td>
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<td>0.62*</td>
<td>0.64</td>
<td></td>
<td>54</td>
</tr>
<tr>
<td>pK_M</td>
<td>2.58</td>
<td>0.32+</td>
<td>0.45</td>
<td></td>
<td>55</td>
</tr>
<tr>
<td>k_0</td>
<td>2.50</td>
<td>0.68</td>
<td>0.25</td>
<td></td>
<td>56</td>
</tr>
<tr>
<td>lr</td>
<td>2.29</td>
<td>-0.22*</td>
<td>0.97</td>
<td>0.4 (.3)</td>
<td>51</td>
</tr>
<tr>
<td>lr</td>
<td>2.27</td>
<td>0.67*</td>
<td>0.96</td>
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<td>57</td>
</tr>
<tr>
<td>pK_I</td>
<td>1.87</td>
<td>0.06*</td>
<td>0.95</td>
<td>0.3 (.2)</td>
<td>58</td>
</tr>
<tr>
<td>V_o</td>
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<td>0.49+</td>
<td>0.90</td>
<td></td>
<td>53</td>
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<tr>
<td>pK_M</td>
<td>1.73</td>
<td>0.20+</td>
<td>0.39</td>
<td>0.4 (.4)</td>
<td>52</td>
</tr>
<tr>
<td>V_o</td>
<td>1.60</td>
<td>0.87</td>
<td>0.87</td>
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<td>59</td>
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<tr>
<td>V_o</td>
<td>1.60</td>
<td>-0.06*</td>
<td>0.99</td>
<td>2.6 (.1)</td>
<td>53</td>
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<tr>
<td>lr</td>
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<td>-0.04*</td>
<td>0.82</td>
<td>0.7 (.3)</td>
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<td>V_o</td>
<td>1.56</td>
<td>-0.13*</td>
<td>0.86</td>
<td>0.3 (.2)</td>
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<tr>
<td>V_m</td>
<td>1.52</td>
<td>0.36</td>
<td>0.13</td>
<td></td>
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<tr>
<td>pK_M</td>
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<td>0.43</td>
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<td>52bc</td>
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<tr>
<td>lr</td>
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<td>0.11*</td>
<td>0.92</td>
<td>0.2 (.2)</td>
<td>60</td>
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<tr>
<td>pD_2</td>
<td>1.30</td>
<td>0.94+</td>
<td>0.89</td>
<td></td>
<td>63</td>
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</table>
Table 2, continued

<table>
<thead>
<tr>
<th>E.A.C.</th>
<th>Description of activity, enzyme and substrate series</th>
</tr>
</thead>
<tbody>
<tr>
<td>125</td>
<td>Inact. of acetyesterase by PNP phosphothionates [0;5]</td>
</tr>
<tr>
<td>126</td>
<td>$\alpha$-CT hydrol. of a.a. PNP esters [0;3]</td>
</tr>
<tr>
<td>127</td>
<td>$\alpha$-CT hydrol. of Z-a.a. PNP esters [0;7]</td>
</tr>
<tr>
<td>128</td>
<td>Inh. of cholinesterase by piperidinium salts [E;4]</td>
</tr>
<tr>
<td>129</td>
<td>Carboxypeptidase-A hydrol. of [4]-altered Ala peptides [E;3]</td>
</tr>
<tr>
<td>130</td>
<td>Renal acylase-Ia hydrol. of acetyl a.a. [0;7]</td>
</tr>
</tbody>
</table>

In order to save space the E.A.C.'s 131 - 144 with more than two pairs in their sets (Ref. 72 - 79) are excluded from this table; they are

RESULTS OF THE SURVEY

In table 3 is given a synopsis of all data treated. The following conclusions may be drawn:

1. Significant correlations were found for 59 of the 101 sets ($n \geq 3$) examined. These included 69% of the total of 700 pairs.
2. In these, E.A.Q.'s greater than zero were as common as values near zero. Negative values occur rarely. Of the sets with zero slopes only 1/3 differed significantly from zero E.I.
3. The non-significant three-point sets together with the two-point sets as a whole reflect a similar distribution, namely a preponderance of positive E.A.Q.'s, frequent occurrence of zero E.A.Q. and infrequently negative values.

As might have been expected, tables 1 and 2 show that significant correlations are found mainly when a large log Eu span is covered. This is in fact perfectly understandable when all the complexities involved are kept in mind. Conversely therefore, it should not be surprising that no significant correlation is seen when this condition
in their sets (Ref. 66 - 72) and the E.A.C.'s 145 - 161 with two pairs however included in table 3.

is not fulfilled. This is clearly borne out by the data which show that most of the sets covering small log Eu spans (e.g. 1.00 or less) show no correlation.
Table 3  Synopsis of Tables 1 and 2.

<table>
<thead>
<tr>
<th>Numbers of pairs in set = n</th>
<th>Significance</th>
<th>E.A.Q. Ave. E.I.</th>
<th>Number of sets</th>
<th>Number of sets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>group 1</td>
<td>group 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total (%)</td>
<td>group 1</td>
</tr>
<tr>
<td>n ≥ 3</td>
<td>Significant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥ 0.3</td>
<td>12</td>
<td>15</td>
<td>27 (26.5)</td>
</tr>
<tr>
<td></td>
<td>≤ -0.3</td>
<td>2</td>
<td>1</td>
<td>3 (3.0)</td>
</tr>
<tr>
<td></td>
<td>-0.3-0.3 {&lt; 0</td>
<td>10</td>
<td>10</td>
<td>20 (19.8)</td>
</tr>
<tr>
<td></td>
<td>≠ 0</td>
<td>4</td>
<td>5</td>
<td>9 (8.9)</td>
</tr>
<tr>
<td></td>
<td>Subtotal</td>
<td>28</td>
<td>31</td>
<td>59 (58.4)</td>
</tr>
<tr>
<td></td>
<td>Non-significant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥ 0.3</td>
<td>21</td>
<td>11</td>
<td>32 (31.7)</td>
</tr>
<tr>
<td></td>
<td>≤ -0.3</td>
<td>1</td>
<td>1</td>
<td>2 (2.0)</td>
</tr>
<tr>
<td></td>
<td>-0.3-0.3</td>
<td>7</td>
<td>1</td>
<td>8 (7.9)</td>
</tr>
<tr>
<td></td>
<td>Subtotal</td>
<td>29</td>
<td>13</td>
<td>42 (41.6)</td>
</tr>
<tr>
<td></td>
<td>Totals for sets with n = 3</td>
<td></td>
<td>57</td>
<td>44</td>
</tr>
<tr>
<td>n = 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥ 0.3</td>
<td>13</td>
<td>6</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>≤ -0.3</td>
<td>6</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>-0.3-0.3</td>
<td>12</td>
<td>8</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Totals for sets with n = 2</td>
<td></td>
<td>31</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Totals for all sets</td>
<td></td>
<td>88</td>
<td>61</td>
</tr>
</tbody>
</table>
The 700 pairs were also examined to see if any general trends are evident. A plot on a common affinity scale is obviously impossible, but all sets have in common a point on the E.I. scale, namely that at which E.I. is zero. The corresponding log Eu value can be calculated from the equation

\[ \text{E.I.} = a + (\text{E.A.Q.}) (\log \text{Eu}) \]

to be

\[ \log \text{Eu}_o = -\frac{a}{\text{E.A.Q.}} \]

where \( a \) is the intercept of the line on the E.I. scale (see Fig. 1c). By translating all the points sideways on the log Eu scale by an amount equal to \( \log \text{Eu}_o \), they can all be plotted together. Such a plot in effect asks what is the average increase in the E.I. for a given increase in affinity. Fig. 9 is a plot of some 200 points belonging to the 30 sets of the total of 101 sets which show a significant correlation to lines of non-zero slopes i.e. with E.A.Q. \( \neq 0^* \).

The best line through all the points is

\[ \text{E.I.} = 0.56 + 0.09 + 0.42 + 0.03 \log \text{Eu} \]

\[ n = 199, \quad r^2 = 0.434, \quad P < 0.1\%. \]

The mean E.A.Q. is significantly different from 1, the value predicted by the Easson-Stedman model (see Part 2).

\* The points of the non-significant correlations or those of E.A.Q. about zero could not be included because the uncertainty of their calculated intercepts is so great that inclusion is meaningless and would be misleading.
Fig. 9. Pooled plot of all sets with significant correlation and E.A.Q. significantly different from zero.

\[ E.I = 0.56 + 0.09 + 0.42 + 0.03 \log Eu; \eta \geq 0.434; \quad P < 0.1\% . \]

**AGONISTS AND ANTAGONISTS**

Group 1 includes many sets that do have a well-defined scale, namely the pharmacological studies in which \( \text{pD}_2 \) or \( \text{pA}_2 \) values are given. For competitive antagonists the \( \text{pA}_2 \) values represent true affinity constants. The same holds true for the \( \text{pD}_2 \) value of partial pharmacological agonists. In case of receptor reserve (which implies that only a fraction of the receptors has to be occupied to obtain a maximal response) the \( \text{pD}_2 \) values will not represent affinity constants directly.

Fig. 10a shows a common plot for 41 points of 9 sets of agonists and Fig. 10b for 102 points of 18 sets of antagonists. Points belonging to non-significant correlations and two-point sets are included.

It can be seen that although similar, the E.A.Q. for agonists is somewhat lower than that for antagonists, although not significantly so \((P = 16\%)\). More important though is the difference of the intercepts of the lines \((\log Eu_0)\): for agonists at a \( \text{pD}_2 \) value of 2.44 and for antagonists at a \( \text{pA}_2 \) value of 5.15 \((P \text{ about } 5\% \text{ that they are equal})\).

From this it can be concluded that (a) the E.A.Q. of a receptor in general is somewhat higher for antagonists and (b) that the same
E.I. is achieved only at much higher (about 630 fold) affinities for antagonists than for agonists. This confirms the generality of the same observations made on one system (Fig. 4, plots a and b).

Fig. 10. Pooled plots of pharmacological systems on their common log Eu scales.

a. $pD_2$ values of agonists

$$E.I. = -0.60 + 0.41 + 0.25 + 0.06 \log Eu; \log Eu_0 = 2.44$$

$$n = 41; r^2 = 0.395; \ P < 0.1\%.$$

b. $pA_2$ values of antagonists

$$E.I. = -1.89 + 0.60 + 0.37 + 0.06 \log Eu; \log Eu_0 = 5.15;$$

$$n = 102; r^2 = 0.275; \ P < 0.1\%.$$

GLOBAL STEREOSELECTIVITY

In Fig. 11 is shown a pooled plot of nearly 700 points belonging to all the sets in groups 1 and 2. It confirms the generality and validity of eudismic-affinity correlations.
After translation to a common origin (as in Fig. 9), the 150 sets, comprising a total of 700 points, were plotted together. The figure shows its central portion and includes some 90% of these points. Note the definite skewness of the distribution. (No special significance should be attached to the points falling far off center; their position is an artifact due to the uncertainty of the calculated intercepts).

**EUDISMIC-AFFINITY CORRELATIONS AS A GENERAL PHENOMENON**

Especially noteworthy is the fact that the correlations are not restricted to chiral pairs, but are also found for prochiral molecules such as geometric isomers. In fact they have also been detected in drug groups where the eutomeric and distomer series are not isomeric but homologous; for example for eudismic pairs of tertiary and quater-
nary (methyl) ammonium derivatives (87), and for eudismic pairs of antihistamines with phenyl and para-chlorophenyl moieties (89).

CONCLUSIONS

We can summarize the salient features of eudismic-affinity correlations as follows:

1. They are observed for very diverse systems, indicating a general occurrence;

2. The quality of a correlation improves
   a. as the span covered by the log Eu values increases
   b. when relative affinity and not absolute configuration are considered;

3. Eudismic-affinity quotients are
   a. usually positive but may also be zero, and occasionally negative
   b. when positive, they are usually less than one
   c. may be different for different receptors interacting with the same homologous series
   d. may be different for different homologous series at the same receptor;

4. Achiral lower homologs may also be correlated although not always;

5. For the same receptor agonists and antagonists have similar E.A.Q.'s but a given E.I. is achieved only at much higher affinities for the antagonists (higher log Eu values).

In conclusion then, on the basis of these data, it would seem that:

1. Stereoselectivity does not occur randomly but is often correlated to activity, respectively, affinity;

2. Most often stereoselectivity increases with affinity, but not always; it often remains constant and occasionally decreases;

3. Correlations occur widely and may be expected under given circumstances in the interaction of chiral or prochiral small molecules with geometrically defined sites (active sites, receptors) on biomacromolecules.

4. The general occurrence of this phenomenon certainly warrants
further investigation along the lines indicated and an analysis of the underlying principle. An interpretation of the observed correlations on the basis of a thermodynamic analysis of a generalized Easson-Stedman model for such interactions will be presented in Part II.

ACKNOWLEDGEMENT

It is a pleasure to acknowledge here the valuable assistance of Dr. R. de Graaf and Drs. W.H. Doesburg in the statistical analysis, Mr. C.P. Nicolasen for the figures and Miss J. Bonnema for secretarial assistance.
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STEREOSELECTIVITY AND AFFINITY IN MOLECULAR PHARMACOLOGY

2. A MOLECULAR BASIS FOR EUDISMIC-AFFINITY CORRELATIONS

J.F. Rodrigues de Miranda*, P.A. Lehmann F.† and E.J. Ariëns
Pharmacology Institute, University of Nijmegen
Nijmegen, The Netherlands.

*On Sabbatical Leave from the Departamento de Farmacología y
Toxicología, Centro de Investigación y Estudios Avanzados,
Shortly after his resolution of the enantiomeric tartaric acids, Pasteur (1) observed that only one of them was metabolized by a mold. Some years later Piutti (2) reported that L-asparagine was tasteless in contrast to the naturally occurring D-asparagine which has a distinct sweet taste. This was another confirmation of Pasteur's (3) brilliant deduction that such phenomena must involve diastereomeric interactions between the enantiomers and a disymmetric receptor site. Numberless further examples of stereospecific phenomena have been found subsequently. This led to the generally accepted view that stereospecificity is a characteristic of life processes.

From pharmacological studies, however, a different picture emerged: in many enantiomeric pairs both isomers were found to have definite, but different, potencies (cf. Beckett's review, 4). Occasionally also, enzymes were found to exhibit such stereoselectivity (Ammon, 5).

A considerable step forward in our comprehension of these observations was made possible with the model introduced by Easson and Stedman (6) to explain the relative potencies of enantiomers. They postulated that the receptor resembles a surface (rather than a cleft) in which three points disymmetrically and rigidly disposed interact with chiral agents. If one isomer has three groups interacting with three points on the receptor site then its enantiomer can only interact with a maximum of two of these. They adduced impressive evidence in favor of this model by showing that 1) removal of one of the three groups led to an achiral analog whose activity was similar to that of the weaker isomer, and 2) that replicating one of the interacting groups to produce another, also achiral, analog re-established activity as expected. A further evaluation of the three-points-interaction model was performed by Niemann et al. (7), who considered the possibility of "wrong-way binding", i.e. alternate binding modes, and attempted to determine "microscopic association factors" for each interaction of every possible binding mode in the particular case of chymotryptic action.

The observations of stereoselectivity cited above, refer to the interaction of more or less closely related series of substrates or drugs with their enzyme or pharmacological receptor. The remarkable observation of Pfeiffer (8) called attention to the wide occurrence of
stereoselectivity. For 14 different drugs he found an excellent correlation between the enantiomeric potency ratio and potency (average human therapeutic dose). This and the observations by Ariëns et al. (9) suggest that we are dealing with quite a general phenomenon (10). To check whether this can be confirmed, the literature has been systematically searched for biological activities of stereoisomers. The results have been given in the preceding paper (11). It presents a survey of available data on the biological activities and enzymological susceptibilities of stereoisomers, and shows that stereoselectivity does not occur randomly but is, in general, a function of the potency or affinity of the more potent isomer.

In this second part an attempt is made to trace the general principle which seems to be underlying the observed correlations between the enantiomeric potency ratio and potency. On the basis of the concepts introduced by Easson and Stedman (6) and Niemann et al. (7), a binding model will be developed which rationalizes those eudismic correlations in which the affinity constant can be considered as a measure of the potency. Special attention will be paid to the different slopes (obtained by plotting the enantiomeric potency ratio versus the potency), encountered in part 1.
The usual description of the pharmacon-receptor interaction:

\[ [F] + [R] \xrightarrow{k_1 \cdot k_{-1}} [RF] \rightarrow \text{effect} \]  

(1)

\[ [RF] = K [R][F] \quad \text{where} \quad K = \frac{k_1}{k_{-1}} \]  

(2)

does not offer a basis for dealing with eudismic interactions. In order to do this it is necessary to consider explicitly individual group contributions to the overall affinity constant \( K \) (cf. the "microscopic binding factors" of Niemann et al., 7).

The stereoselectivity of biological activity observed for stereoisomers must be based on diastereomeric interactions between chiral or prochiral molecules and a disymmetric site on the macromolecular receptor molecule or enzyme. Such interactions necessarily imply that the macromolecule has three or more points of attachment for which select types of molecules show high affinity. These points may lie in a plane or not (e.g. the cleft in lysozyme) and may be symmetrically disposed, or not, about a point which can be designated as the chiral center of the FR-complex.

We will discuss the subject by dealing with the most general case of a four-point chiral arrangement symmetrically (tetrahedral) disposed in space. To facilitate the discussion the following definitions introduced in part 1 will be used:

In a homologous series of stereoisomeric pairs (enantiomeric or diastereomeric, such as geometric or epimeric) the stronger and weaker of each pair are designated eutomer (Eu) and distomer (Dis) respectively, independently of their absolute configuration.

Since the description given below is limited to those cases in which the affinity constant, \( K \), can be considered as a measure of the potency, \( \log \) (potency) of the eutomer is designated as \( \log K_{Eu} \) (contrary to part 1, in which it was designated as \( \log Eu \)). The ratio of the potencies of the isomers is defined as the eudismic ratio (E.R.), while its logarithm is defined as the eudismic index (E.I.). Finally, the change of E.I. per unit change in \( \log K_{Eu} \) is termed the eudismic-affinity quotient (E.A.Q.); it is a quantitative estimate of stereoselectivity.
Consider a rigid receptor with four sites (A, B, C, D) so disposed spatially as to interact optimally with one enantiomer of a tetrahedral molecule bearing four groups (a, b, c, d) about a chiral atom such as C or P. Keeping one group fixed (for example d on D in Fig. 1), it can be seen that three different arrangements are possible for each enantiomer. Since any of the four groups could be fixed at D, there are a total of twelve arrangements possible for each enantiomer. These can be described in terms of the individual contacts; for example one is Aa Bb Cc Dd.

Fig. 1. Possible arrangements of the R (above) and S (below) forms of a chiral agent with a tetrahedral receptor of chirality complementary to the R-form. When one interaction is fixed (d on D) there are three possible arrangements for each enantiomer. Congruences are underlined.
All 24 arrangements are conveniently handled in terms of the permutations of an $4 \times 4$ matrix (7). The 12 permutations with an even number of inversions defining them as positive correspond to the eutomer arrangements and the 12 permutations with an odd number of inversions (negative signs) to those of the distomer (see table 1).

Designating contacts between site and group bearing the same letter as congruences, it can be seen that the eutomer evinces one quadruple and 8 single congruences, whereas the distomer has only 6 double congruences. Note that three-point contacts, i.e. tridentate complexes do not exist for the eutomer; likewise for the distomer monodentate complexes are absent.

BINDING ASPECTS

Let us consider a stepwise formation of the tetridentate complex via mono-, bi- en tridentate intermediates:

\[
\begin{align*}
A & \overset{a}{\rightleftharpoons} B + \overset{b}{\rightleftharpoons} C + \overset{c}{\rightleftharpoons} D \overset{d}{\rightleftharpoons} F
\end{align*}
\]

then the concentration of each complex can be written (in simplified notation) as:

\[
\begin{align*}
[R=F] &= k_{Aa} [R] [F] \\
[R=F] &= k_{Aa} k_{Bb} [R] [F] \\
[R=F] &= k_{Aa} k_{Bb} k_{Cc} [R] [F] \\
[R=F] &= k_{Aa} k_{Bb} k_{Cc} k_{Dd} [R] [F]
\end{align*}
\]

where $k_{i}$ are group-affinity constants for the individual steps. Below they will be replaced by $k_{i}$. The designations $k_{a}, k_{b}, k_{c}, k_{d}$ do not refer to particular substituents but to a ranking which is chosen as
### Table 1
Tetrahedral diastereomeric arrangements

<table>
<thead>
<tr>
<th></th>
<th>Aa</th>
<th>Ba</th>
<th>Ca</th>
<th>Da</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab</td>
<td>Bb</td>
<td>Cb</td>
<td>Db</td>
<td></td>
</tr>
<tr>
<td>Ac</td>
<td>Bc</td>
<td>Cc</td>
<td>Dc</td>
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</tr>
<tr>
<td>Ad</td>
<td>Bd</td>
<td>Cd</td>
<td>Dd</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Factorization</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aa Bb Cb Db</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aa Bd Cc Dd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aa Bd Cb Dd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aa Bc Cd Db</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aa Bb Cc Pb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aa Bb Cd De</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aa Bc Cb Dd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I Ba Ca Da</td>
<td>Ab Ba Cc Dd</td>
<td></td>
</tr>
<tr>
<td>I Bd Cd Dd</td>
<td>Ab Bd Ca Dc</td>
<td></td>
</tr>
<tr>
<td>I Bd Cd Dd</td>
<td>Ab Bc Ca Dd</td>
<td></td>
</tr>
</tbody>
</table>

### Congruences

1 quadruple and 8 single

6 double

\[ k_a > k_b > k_c > k_d. \]

In view of the high affinities normally encountered for pharmacological receptors (\( pA_2 \) or \( pD_2 \sim 6-9 \)) we can safely infer that only multidentate complexes are important, i.e. we can neglect the contributions from single congruences. In this event, as follows from the previous section, only tetradentate complexes for the eutomer and bidentate complexes for the distomer need be considered. Thus we can immediately write down the concentrations of the effective complexes (table 1) of both enantiomers:
\[ \text{Eu} : [R \equiv F] = K_{\text{Eu}} \frac{[R][F]}{[R][F]} = k_a k_b k_c k_d \frac{[R][F]}{[R][F]} \] (8)

and

\[ \text{Dis} : [R \equiv F] = K_{\text{Dis}} \frac{[R][F]}{[R][F]} = (k_a k_b + k_a k_c + k_a k_d + k_b k_c + k_b k_d + k_c k_d) \frac{[R][F]}{[R][F]} \] (9)

**EUDISMIC ASPECTS**

If the pharmacological response is directly proportional to the concentration of effective FR-complexes (neglecting intrinsic activity), the potency ratio, E.R., equals the ratio of the effective complex concentrations and by taking the ratio of (8) over (9).

\[ \text{E.R.} = \frac{[R \equiv F]}{[R \equiv F]} = \frac{K_{\text{Eu}}}{K_{\text{Dis}}} = \frac{k_a k_b k_c k_d}{k_a k_b + k_a k_c + k_a k_d + k_b k_c + k_b k_d + k_c k_d} \] (10)

It can be seen that eutomer potency is a fourth-order term whereas distomer potency is the sum of six second-order terms. Thus both are increasing functions of \( k_i \), but \( K_{\text{Eu}} \) will increase much faster than \( K_{\text{Dis}} \). It can easily be shown that, if - within a series of compounds - an increase of one group-affinity constant is not associated with a decrease of another, E.I. is a positive increasing function of \( \log K_{\text{Eu}} \).

**EUDISMIC RELATIONS**

All observed eudismic relations (Part 1) are contained implicitly in eq. 10. Because of its complex nature certain reasonable simplifying assumptions will be made. These allow the derivation of simple explicit expressions for E.A.Q. which can be used to test the above model against the experimental data of Part 1.

Our main assumption is that \( k_a \gg k_c \). Then we can neglect in eq. 10 all the terms in the denominator containing \( k_c \) and \( k_d \), and write:
This means that within this approximation stereoselectivity is determined by the two lower binding constants.

What we are pharmacologically interested in is the change of E.I. within a series of compounds. To this end we compare two pairs of isomers.

\[
\begin{align*}
\text{pair 1:} & \quad \frac{K_{\text{Eu}(1)}}{K_{\text{Dis}(1)}} = k_{c1} k_{d1} = \text{E.R.}(1) \\
\text{pair 2:} & \quad \frac{K_{\text{Eu}(2)}}{K_{\text{Dis}(2)}} = k_{c2} k_{d2} = \text{E.R.}(2)
\end{align*}
\]

If we take:

\[K_{\text{Eu}(2)} > K_{\text{Eu}(1)}\]

it then holds that:

\[
\text{E.A.Q.} = \frac{\text{E.I.}(2) - \text{E.I.}(1)}{\log K_{\text{Eu}(2)} - \log K_{\text{Eu}(1)}} = \log k_{c2} k_{d2} - \log k_{c1} k_{d1}
\]

or replacing \(\log k_c k_d\) by \(\log K_{\text{Eu}} - \log k_a k_b\)

\[
\text{E.A.Q.} = 1 - \frac{\log k_{a2} k_{b2} - \log k_{a1} k_{b1}}{\log K_{\text{Eu}(2)} - \log K_{\text{Eu}(1)}}
\]

Two broad divisions in relation to the E.A.Q. values can be distinguished.
If it is assumed that
\[ k_{a1} k_{b1} < k_{a2} k_{b2} \]
and also that
\[ k_{c1} k_{d1} < k_{c2} k_{d2} \]
then
\[ \log k_{a2} k_{b2} - \log k_{a1} k_{b1} < \log K_{Eu(2)} - \log K_{Eu(1)} \]
According to eq. 14 it follows that:
\[ 0 < E.A.Q. < 1 \]
Physically this means that, within a series of pairs of isomers, changes in the molecular structure are such, that with the increase of \( K_{Eu} \), both the product of the two lower and the two higher affinity constants increase. In this case \( E.I. \) is an increasing function of \( \log K_{Eu} \) with \( 0 < E.A.Q. < 1 \) such as shown in Fig. 2a. Within this division the following limiting situations can be considered:

**Situation 1**

\[ k_{a1} k_{b1} = k_{a2} k_{b2} \]

i.e. the product \( k_a k_b \) does not change within the series of isomers considered. Then according to eq. 14:

\[ E.A.Q. = 1 \]
Physically this means that within a series of sets of isomers changes in the molecular structure are limited to those parts of the molecule which only contribute to \( k_c \) and \( k_d \), but not to the product of \( k_a \) and \( k_b \). In this case \( E.I. \) is an increasing linear function of \( \log K_{Eu} \) with an \( E.A.Q. \) of 1, such as shown in Fig. 2b. Examples of this case are found in Figs. 7 and 8 of part I.
Fig. 2. Some typical eudismic relations. Possible correlations between the affinities of a homologous series of enantiomers can be sought in two ways:

i (above): Both the affinity of the eutomer (●) and that of the distomer (▲) are plotted against that of the eutomer; the line joining the squares obviously has a slope of 1, but the drop in affinity for the distomer is apparent.

ii (below): Here the same data have been plotted in a more compact form; i.e. E.I. (= log \( K_{Eu} \) - log \( K_{Dis} \)) vs log \( K_{Eu} \) (●). The slopes of the lines (E.A.Q) correspond to the four limiting cases of eudismic relations considered.

Situation 2

\[
\begin{align*}
  k_{c1}k_{d1} &= k_{c2}k_{d2} \\
\end{align*}
\]

i.e. the product \( k_{c1}k_{d1} \) does not change within the series of isomers considered. Then according to eq. 12:

\[
E.I. (2) - E.I. (1) = 0 \quad \text{and} \quad E.A.Q. = 0
\]

Physically this means that within a series of sets of isomers changes in the molecular structure are limited to those parts of the molecule which only contribute to \( k_a \) and \( k_b \) but not to the product of \( k_c \) and \( k_d \). In this case \( E.I. \) is constant and \( E.A.Q. = 0 \) as shown in Fig. 2c.

Examples of this situation are found in Fig. 2 and E.A.C. ¶ 3 and 26 of table 1 of part I.

Within the latter situation only two cases can be distinguished.
a) E.I. is zero, i.e. there is no stereoselectivity. This can occur if only two groups are effectively interacting, the third and fourth groups either will not interact at all (the receptor has only two sites) or their binding contributions to existent third and fourth sites are negligible.

b) E.I. is constant but different from zero, i.e. the series displays constant stereoselectivity. This can occur if only $a$ and/or $b$ are being varied and groups $c$ and $d$ remain unchanged throughout the series, i.e. $E.I. = \log k_c + \log k_d =$ constant.

**Division II**

If it is assumed that

\[ k_{a1} k_{b1} < k_{a2} k_{b2} \]

and also that

\[ k_{c1} k_{d1} > k_{c2} k_{d2} \]

then

\[ \log k_{a2} k_{b2} - \log k_{a1} k_{b1} > \log K_{Eu(2)} - \log K_{Eu(1)} \]

According to eq. 20 it follows that:

\[ E.A.Q. < 0 \]

Physically this means that within a series of pairs of isomers, changes in the molecular structure are such that part of the increase in $K_{Eu}$ as caused by the groups with the largest contribution to the overall stability of the complex is lost by a decrease in the contributions of the other groups. In that case $E.I.$ is a decreasing function of $\log K_{Eu}$ with $E.A.Q. < 0$, such as shown in Fig. 2d. This case is the special one which was excluded from the general consideration in relation to eq. 10. Examples of this case are found in Fig. 8 and FAC ¶ 23 of table 1 of part 1.
DISCUSSION

GENERAL OCCURRENCE OF EUDISMIC-AFFINITY CORRELATIONS

The model for drug-receptor interaction, as described before, shows the general trends for stereoselectivity discussed in part 1 (11). Remarkably, this even holds true if for the group affinity constants particular assumptions are made such as $k_a >> k_c$, while then also the limiting situations $E.A.Q. = 1$ and $E.A.Q. = 0$ evolve. This is partly inherent in the way stereoselectivity is treated here, i.e. plots of the interrelated variables $E.I.$ and $\log K_{Eu}$ limit the number of possible slopes. Nevertheless it implies, as shown in equation 12, that stereoselectivity is determined by those group affinity constants which make the lowest contribution to the overall affinity constant. Since the condition $k_a >> k_c$ will often be fulfilled, the general occurrence of eudismic affinity correlations can be explained on this basis. In part 1 a few cases were encountered with an $E.A.Q.$ greater than unity. The model used does not predict the existence of such a relation. It is likely that in such cases these $E.A.Q.$ values are determined by steps subsequent to binding. In fact, the above mentioned cases all involve the correlation of enzymatic turnover data.

GROUP AFFINITY CONSTANTS

The usefulness of the model presented would increase greatly if the group affinity constants utilized could be interpreted as intrinsic group affinity constants like those used in the correlation of reaction rates (12, 13), or biological activity (14, 15), with physico-chemical parameters. These would be constant throughout different drug series and biological systems, enabling the prediction of stereoselectivity. In fact we (Lien et al., 16) have shown that the stereoselectivity of a series of phenoxypropionic acids acting as plant growth regulators can be explained if the physicochemical constants of the two chiral substituents are treated as independent variables.

In the determination of the group affinity constants, defined above, a number of complications will, however, be encountered. First of all it has to be assumed that the observed biological activity depends linearly on the affinity. In neglecting intrinsic activity and $k_3$ for enzymatic reactions for the moment, the next difficulty encountered concerns the process of binding of a molecule to its binding site or
receptor, which may be quite complicated. From a number of kinetic studies of substrate binding to proteins (17-19), evidence is accumulating that often association rate constants are not diffusion-limited. This has been interpreted to mean that in this type of binding process a pre-equilibrium condition is established before all interacting groups become attached. Other work (20, 21) has shown that in multistep complex formation, involving identical groups, nucleation and propagation steps can be distinguished. In general, once nucleation has occurred, propagation is rather easy and similar for all subsequent interactions. Another point which has to be taken into account is that the sequence of formation of $R\equiv F$, indicated in eq. 3, is not the only possible one; other sequences such as $a \rightarrow c \rightarrow b \rightarrow d$ might be considered as well. Although the overall affinity constants $K_{Eu}$ and $K_{Dis}$ are, of course, independent of the sequence followed, one should question whether the individual group affinity constants are equal for each sequence and whether, for example, $k_b$ in eq. 8 has the same value as $k_b$ in eq. 9.

If however, the sequence of complex formation can be described by a nucleation step and a propagation step, and if the nucleation step is confined to the first interacting group (a or b) then at least the value of the product of $k_a k_b$ in the numerator and denominator of eq. 11 will be the same and independent of the reaction sequence. This makes the assumption that eq. 12 is independent of the sequence of binding steps quite reasonable. Moreover, as eq. 12 states that stereoselectivity is determined only by $k_c$ and $k_d$, and if, as is assumed above, the corresponding groups are not involved in nucleation, it is quite possible that they might turn out to be adequate substituent constants, useful in predicting stereoselectivity.

The model presented is simple and adequate to explain the relation of stereoselectivity and affinity in general. It is not possible yet, however, to correlate the group affinity constants of the model to the particular groups in the stereoisomers.
SUMMARY AND CONCLUSIONS

In part I of these two papers (11) experimental evidence is given for particular relationships between the ratio of the activities (potencies) of pairs of optical isomers in relation to the activity of the more potent isomer of the pair. This ratio in general increases with the activity of the more potent isomer. This relationship occurs among such a wide variety of bioactive systems (see part I) that a general principle underlying the phenomenon becomes probable.

In this part, on the basis of a simplified drug-receptor interaction model and with emphasis on the contributions to the overall affinity by the various (four) ligands at the chiral center, this general principle has been rationalized. The relations observed experimentally are explained in terms of the group affinity constants involved. The model predicts the prevalence of a positive correlation between the ratios of activities of the pairs of isomers and the activity of the more potent isomer.

Further general trends that follow from the analysis given are:

1. The slope of the curve representing the relationship will not exceed unity when only affinities are correlated.
2. A negative value for the slope of the curve representing the relationship is only to be expected under particular conditions, namely if, with the increase in the affinity of the more active isomer, there is a concomitant and larger increase in the affinity of the less active isomer such that the latter still remains less active than the first. This implies that such a relationship will only occur over a small span of activities.

The analysis presented also indicates that it should be possible to obtain "substituent" constants useful in predicting stereoselectivity. The experimental data required for such a procedure are not sufficiently available yet.

ACKNOWLEDGEMENT

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REFERENCES

When designing new drugs the aim should be to obtain a drug with optimal selectivity rather than high activity. In this way unwanted side effects will be avoided. In general a high selectivity of a drug will be based on a high degree of complementarity of the chemical properties of the drug and the site of interaction in the body - the receptor - where the desired effect is induced. Therefore, this complementarity must be as complete as possible: the interaction must have a high degree of specificity. The objective of the study described in this thesis is to contribute to the insight in the factors determining the specificity of the interaction between the drug and its receptor.

Section I deals with the question in how far the hydrophobic interaction between a part of the pharmacon molecule and a part of the receptor can play a role in the specificity. In the literature a correlation between activity and lipophilicity inside series of bioactive agents is reported quite often. The partition coefficient is then commonly used as a measure for the lipophilicity. However, since a great diversity of drugs is concerned the significance of the lipophilicity in the selectivity of the interaction seems to be small. A lipophilicity parameter such as the partition coefficient or \( \pi \) gives only an indication of a general property and does not express its spatial aspects. Such a parameter therefore is unsuitable for approaching the selectivity in action. For those cases in which the receptor is located on a protein it can be expected - taking into account the structure of proteins - that the hydrophobic binding areas are small. Thus it seems probable that hydrophobic binding can play a role in the selectivity. This problem has been investigated experimentally by determining the affinity constants for the binding to albumin of a series of successive fatty acids (propionic acid to nonanic acid) at two temperatures. The values of the standard free energy change, \( \Delta G^0 \), for the binding to the specific binding site, as derived from the affinity constants, do not increase linearly with the increase in chain length. Between valeric acid and heptanoic acid the values show a plateau. The occurrence of the plateau can be interpreted as being the consequence of a restricted size of the hydrophobic binding area of albumin for the fatty acids. A similar discontinuity in the dependence of \( \Delta G^0 \) on the chain length has been observed for other protein systems. Therefore it is probable that a re-
stricted size of hydrophobic binding areas is a general feature of proteins. This knowledge could be employed to increase the selectivity in action of bioactive agents.

Section II reports the study of the binding of acetrizoate to albumin by means of nuclear magnetic resonance (nmr). Acetrizoate shows a pronounced selectivity with regard to the various binding sites of albumin. The ratio of the affinity constants of the first and remaining binding sites is about 1000. The nmr signals show a linewidth increase and a shift as a result of the binding. These changes in the spectrum are determined by the exchange rate of the pharmacon molecule between bound and not-bound state, by the mobility of the various groups of the molecule and the active site on the receptor, and by their mutual distances. From the linewidth measurements, values could be determined for the association and dissociation rate constants of the two types of complexes. It appears that an increase of the affinity constant is much more coupled with an increase of the association rate constant, rather than with a decrease of the dissociation rate constant. The specific interaction shows a much higher association rate constant \(2.5 \times 10^7 \text{ sec}^{-1} \text{ M}^{-1}\) than the non-specific one \(2 \times 10^7 \text{ sec}^{-1} \text{ M}^{-1}\). Obviously, neither of the two rate constants are diffusion limited. The influence of the dissociation rate constant of the specific complex on the efficiency of renal excretion has been checked in model calculations.

The increase in linewidth provides information about the mobility of the groups of the molecule in the bound state. In the case of the non-specific complex, this information has been used to estimate the mobilities of the methyl group and the phenyl proton. Both groups in this complex show residual mobility with regard to the albumin molecule.

Section III deals with the difference in biological activity of stereoisomers. The fact that these differences occur emphasizes the role of the spatial aspects in the selectivity of interaction. A difference in activity of enantiomers is really only explicable if at the interaction site of the more active isomer at least three, spatial separated, interaction points are involved. A literature survey of relevant experimental data shows that often a remarkable relationship exists between the ratio of the activities of the enantiomers on one side and the activity of the more active isomer on the other. This leads to the expectation of a general basic principle underlying this phenomenon.
The observed correlations can be rationalized on the basis of a simple theoretical model for diastereomeric interactions. Emphasis is laid on the individual contributions of the groups, attached to the chiral carbon atom, to the overall affinity of the drug molecule to its receptor.
S/WENVATTING

Bij het ontwerpen van nieuwe geneesmiddelen is het niet primair van belang om hoog werkzame verbindingen te verkrijgen maar veel meer om de selectiviteit van het geneesmiddel zo optimaal mogelijk te maken. Men vermijdt hierdoor het optreden van ongewenste bijwerkingen. Aangezien de selectiviteit van het geneesmiddel in de regel zal berusten op een hoge mate van complementariteit van de chemische eigenschappen van het geneesmiddel met die van de plaats van interactie in het lichaam, de receptor, al waar het gewenste effect wordt geïnduceerd, zal deze complementariteit zo goed mogelijk moeten zijn; de interactie zal een hoge mate van specificiteit moeten bezitten. Het is het doel van de onderzoekingen, beschreven in dit proefschrift, om het inzicht te verrijken in de factoren die de specificiteit van de interactie van het geneesmiddel met zijn receptor bepalen.

In sectie I wordt nagegaan in hoeverre de hydrofobe interactie van een deel van het farmacomolecuul met een deel van de receptor een rol kan spelen in de specificiteit van de interactie van het farmacomolecuul met zijn receptor. In de literatuur wordt veelvuldig melding gemaakt van een correlatie tussen werkzaamheid en lipofiliteit binnen reeksen van bioactieve stoffen. Hierbij wordt de verdelingscoëfficiënt in de regel als maat voor de lipofiliteit gehanteerd. Daar het hier een grote diversiteit van verbindingen betreft lijkt de betekenis van de lipofiliteit in de selectiviteit van de interactie gering. Een lipofiliteitsparameter, zoals verdelingscoëfficiënt of π, geeft echter slechts een algemene hoedanigheid weer en geeft geen uitdruking aan de ruimtelijke aspecten ervan. Een dergelijke parameter is dan ook niet geschikt om de selectiviteit in werking te benaderen. Voor die gevallen dat de receptor gelokaliseerd is op een eiwit kan op grond van de bouw van het eiwit verwacht worden dat de hydrofobe bindingsgebieden beperkt in omvang zijn. Hiervan uitgaande, is het waarschijnlijk dat hydrofobe binding wel een rol kan spelen bij de selectiviteit in werking. Experimenteel is dit vraagstuk aangepakt door het bepalen van affiniteitsconstanten voor de binding aan albumine van een reeks opeenvolgende vetzuren (propionzuur tot nonaanzuur) bij twee temperaturen. De hiervan afgeleide standaardvrije energieverandering, $\Delta C^0$, voor de binding aan de specifieke bindingsplaats neemt, met het langer worden van de vetzuurstaart, niet monotoon toe. Bij valeriaanzuur wordt een plateau in $\Delta C^0$ bereikt.
en pas bij heptaanzuur neemt $\Delta G^0$ verder toe. Het optreden van het plateau in $\Delta G^0$ kan worden gezien als een afspiegeling van de beperkte omvang van het hydrofobe bindingsgebied van albumine voor vetzuren. Aangezien voor andere eiwitsystemen soortgelijke discontinuïteiten in de afhankelijkheid van $\Delta G^0$ van de ketenlengte zijn vastgesteld is het waarschijnlijk dat de beperktheid in de grootte van de hydrofobe bindingsgebieden een algemeen kenmerk voor eiwitten is. Deze kennis zou benut kunnen worden om de selectiviteit in de werking van geneesmiddelen te verhogen.

In sectie II is de binding van acetrizoaat aan albumine met behulp van kerrmagnetische resonantie (nmr) onderzocht. Acetrizoaat vertoont een duidelijke selectiviteit t.a.v. verschillende bindingsplaatsen op het albumine. De affiniteitsconstanten van de eerste bindingsplaats en die van de overige bindingsplaatsen verhouden zich ongeveer als 1000 : 1. De nmr signalen verbreden en verschuiven als gevolg van de binding van acetrizoaat aan albumine. Deze veranderingen in het spectrum worden bepaald door de overgangsnelheid van het farmacon van de gebonden naar de niet gebonden toestand en omgekeerd, de beweeglijkheid van de afzonderlijke groepen in het farmaconmolecuul en de actieve plaats op de recepto, en de onderlinge afstand van deze groepen. Uit de lijnbreedtemetingen konden waarden voor de associatie- en dissociatiesnelheidsconstanten voor de twee soorten complexen worden bepaald. Hieruit blijkt dat een toename in de affiniteitsconstanten niet gepaard gaat met een afname van de dissociatiesnelheidsconstante maar met een toename in de associatiesnelheidsconstante. De specifieke interactie heeft een veel hogere $(2,5 \cdot 10^3 \text{ sec}^{-1} \text{ M}^{-1})$ associatiesnelheidsconstante dan de niet specifieke $(2 \cdot 10^3 \text{ sec}^{-1} \text{ M}^{-1})$ interactie. Beide associatiesnelheden worden duidelijk niet bepaald door de diffusiesnelheid. In modelberekeningen werd de invloed van de dissociatiesnelheid van het specifieke acetrizoaat-albumine complex op de effectiviteit van de renale klaring nagegaan. De informatie die de lijnverbreding biedt omtrent de beweeglijkheden der groepen kon in geval van de niet specifieke interactie worden aangewend om de beweeglijkheden van de methylgroep en het aromaatproton te benaderen. Beide groepen bleken in dit complex nog een residuele beweeglijkheid ten opzichte van het albumine te bezitten.

In sectie III zijn de biologische activiteiten van stereo-isomeren onderling vergeleken. Het optreden van verschillen in werkzaamheid
van enantiomeren accentueert de betekenis van de ruimtelijke structuur voor de selectiviteit in de interactie. Een verschil in werkzaamheid van enantiomeren is immers slechts verklaarbaar indien bij de interactie tussen de meest werkzame isomeer van het farmacon en de receptor minstens drie ruimtelijk gescheiden interactiepunten betrokken zijn. Aan de hand van een literatuuroverzicht van relevante experimentele gegevens wordt aangetoond dat vaak een opvallend verband bestaat tussen de verhouding der activiteiten van de enantiomeren en de activiteit van meest werkzame isomeer. Deze relatie blijkt zo algemeen dat ze pleit voor een algemeen principe als grondslag ervoor. Aan de hand van een eenvoudig theoretisch model voor diastereomere interacties kunnen de waargenomen correlaties verklaard worden. Er wordt hierbij de nadruk gelegd op de afzonderlijke bijdragen van de, aan het asymmetrische koolstofatoom bevestigde, groepen aan de totale affiniteit van het farmaconmolecuul voor zijn receptor.
CURRICULUM VITAE


Onder leiding van Prof. Dr. E.J. Ariëns verrichtte hij onderzoek naar verschillende aspecten van de binding van farmaca aan plasma-eiwitten waaronder ook de invloed van de binding op de renale klaring. Onder leiding van Dr. C.W. Hilbers kon zijn belangstelling voor de toepassingsmogelijkheden van magnetische resonantie ter bestudering van structuurwerkingsmechanismen geëffectueerd worden. Uit gezamenlijk onderzoek zijn de volgende publicaties voortgekomen:

1. The significance of the ester group in acetylcholine.

2. A method for the quantitative determination of the total concentration of radiopaque agents in plasma.

3. pK Change of imidazoline groups in bovine serum albumin due to conformational change at neutral pH.
4. Pharmacokinetics of renal contrast media. I. Renal excretion processes as studied in dogs by the stop-flow technique.

5. Non-exponential relaxation of the methyl protons of acetrizoate in solution.


7. A nmr study of the kinetics of the binding of the renal contrast medium acetrizoate to albumin.
   J.F. Rodrigues de Miranda and C.W. Hilbers, submitted for publication.

8. The kinetics of the binding of acetrizoate to albumin in relation to its renal clearance.
   J.F. Rodrigues de Miranda, M.A. van 't Hof and C.W. Hilbers, submitted for publication.

9. The extent of hydrophobic binding area studied by fatty acid binding to albumin.

10. Stereoselectivity and affinity in molecular pharmacology.
    1. The correlation of stereoselectivity and activity.

11. Stereoselectivity and affinity in molecular pharmacology.
    2. A molecular basis for eudismic-affinity correlations.

I

Kennis omtrent de afzonderlijke bijdragen van groepen in het farmacon-molecuul tot de stabiliteit van het farmacon-receptorcomplex, kan het inzicht in het mechanisme van selectiviteit in werking wezenlijk vergroten. Het verband tussen de temperatuur en de correlatietijden van de afzonderlijke groepen, zoals dat uit de nmr-lijnbreedten is af te leiden, zou een indicatie voor de grootte van deze bijdragen kunnen zijn.


Dit proefschrift.

II

De voorwaarden voor fast exchange, zoals door Granot en Fiat geformuleerd, zijn onderling strijdig.


III

De onderlinge posities en grootten van lipofiele bindingsgebieden, gelegen in een overwegend hydrofobe omgeving op of in eiwitten, kunnen door het bepalen van de binding van een reeks vetzuurderivaten worden onderzocht. Hierbij dient de positie van een polaire groep in de alifatische keten van deze vetzuurderivaten gevarieerd te worden.

Dit proefschrift.

IV

Het experimentele materiaal dat Müller en Wollert aandragen om aan te tonen dat 1-tryptofaan en benzodiazepine-derivaten de eerste specifieke bindingsplaats op albumine gemeenschappelijk hebben, heeft onvoldoende bewijskracht.

Het vervolgen van de vrije concentratie in plaats van de totale concentratie van een geneesmiddel in het plasma kan de farmacokinetische interpretatie van de verdwijningscurve vereenvoudigen.

VI

Schenkman's interpretatie van convex verlopende dubbel-reciproke plots is niet de meest waarschijnlijke.
J.B. Schenkman, Biochem. 9, 2081 (1970).

VII

De waarneming dat, bij interactie van biomacromoleculen met een overmaat substraat een verbreding van de resonantielijnen in het nmr-spectrum niet gepaard gaat met een verschuiving van deze lijnen, wordt door Seydel en Wasserman ten onrechte als een argument tegen een langzame uitwisseling (op nmr-tijdschaal) aangevoerd.
Chimie Thérapeutique 4, 427 (1973).

VIII

Gezien de hoeveelheid moeite en geld die aan de ontwikkeling van psychofarmaca wordt besteed, zou gericht wetenschappelijk onderzoek naar de waarde van alternatieve - psychodynamische - methoden ter bestrijding van stress, zoals meditatie, eveneens krachtig bevorderd dienen te worden.

Nijmegen, 10 oktober 1975

J.F.R. de Miranda