Model studies in bio-organic processes: sodium transport across biological membranes: an experimental study: quantum chemical calculations on the stereochemistry of coenzyme B12 dependent carbon-skeleton rearrangements

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An experimental study.

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Inge Merkelbach
MODEL STUDIES IN BIO-ORGANIC PROCESSES.

Sodium ion transport across biological membranes. An experimental study.

Quantum chemical calculations on the stereochemistry of coenzyme B$_{12}$ dependent carbon-skeleton rearrangements.

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TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE TECHNISCHE WETENSCHAPPEN AAN DE TECHNISCHE HOGESCHOOL EINDHOVEN, OP GEZAG VAN DE RECTOR MAGNIFICUS, PROF. DR. S.T.M. ACKERMANS, VOOR EEN COMMISSIE AANGEWZEZEN DOOR HET COLLEGE VAN DEKANEN IN HET OPENBAAR TE VERDEDIGEN OP VRIJDAG 10 MEI 1985 TE 16.00 UUR

DOOR

INGRID IRENE MERKELBACH

GEBOREN TE MOZAMBIQUE
DIT PROEFSCHRIFT IS GOEDGEKEURD

DOOR DE PROMOTOREN

PROF. DR. H.M. BUCK

EN

PROF. DR. K. VAN DAM
# CONTENTS

**Sodium ion transport across biological membranes.**
**An experimental study.**

## I. **Introduction.**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.1. Membrane properties.</td>
<td>9</td>
</tr>
<tr>
<td>I.2. Membrane function. Scope of the first part of this thesis.</td>
<td>11</td>
</tr>
<tr>
<td>I.3. Developments in organophosphorus chemistry.</td>
<td>14</td>
</tr>
<tr>
<td>I.4. Transfer of conformational changes in a phosphate group to the hydrophobic part of organic molecules.</td>
<td>18</td>
</tr>
</tbody>
</table>

References and notes. 22

## II. **Sodium ion transport across biological membranes.**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>II.1. Theory.</td>
<td>25</td>
</tr>
<tr>
<td>II.1.1. Cluster formation in biological membranes induced via a phospholipid P(V) TBP intermediate.</td>
<td>25</td>
</tr>
<tr>
<td>II.1.2. Activation of membrane proteins by uptake in phospholipid clusters.</td>
<td>33</td>
</tr>
<tr>
<td>II.2. Experiments.</td>
<td>37</td>
</tr>
<tr>
<td>II.2.1. Introduction.</td>
<td>37</td>
</tr>
<tr>
<td>II.2.2. Results.</td>
<td>40</td>
</tr>
<tr>
<td>II.2.3. Discussion.</td>
<td>43</td>
</tr>
<tr>
<td>II.2.4. Conclusion.</td>
<td>50</td>
</tr>
<tr>
<td>II.2.5. Experimental.</td>
<td>50</td>
</tr>
</tbody>
</table>

References and notes. 53
Quantum chemical calculations on the stereochemistry of coenzyme B\textsubscript{12}-dependent carbon-skeleton rearrangements.

III. The formation of a carbanionic intermediate in the carbon-skeleton rearrangement step.

III.1 Structure and function of coenzyme B\textsubscript{12}. 59
III.2 Mechanism of action of the carbon-skeleton rearrangements. 62
III.3 The stereochemistry of the carbon-skeleton rearrangements as 'test' for the carbanionic mechanism. Scope of the second part of this thesis. 65
III.4 The nature of the hydrogen transferred temporarily to coenzyme B\textsubscript{12} during the carbon-skeleton rearrangements. 67

References and notes. 69

IV. Quantum chemical calculations.

IV.1 Introduction. 73
IV.2 The choice of the calculational method. 74
IV.3 Results. 76
IV.4 Discussion. 85
IV.5 Conclusion. 87

References and notes. 88

Summary 89
Samenvatting 91
Curriculum Vitae 93
Dankwoord 94
aan Emmy, aan René
Voor het slagen van het kwaad is niets anders nodig dan dat de goede mensen niets doen.

Maarten Luther King.
Sodium ion transport across biological membranes.
An experimental study.
I. Introduction.

I.1. Membrane properties.

Biological membranes play a crucial role in almost all cellular phenomena, yet our understanding of the molecular organization of membranes still can be called far from exhaustive. While the composition of membranes varies with their source, they generally contain approximately 40% of their dry weight as lipid and 60% as protein. Usually carbohydrate is present to the extent of 1-10% of the total dry weight. In addition to these components, membranes contain some 20% of their total weight as water, which is tightly bound and essential to the maintenance of their

Figure I.1. The fluid mosaic model of Singer and Nicolson. Integral proteins (crossing the lipid bilayer), peripheral proteins (bound to the exterior of the bilayer) and proteins partly embedded in the bilayer matrix are bound to a functional complex or dissolved individually in the membrane bilayer.
structure. These components are organized according to the fluid mosaic model of Singer and Nicolson\textsuperscript{2} (see Figure I.1.). The lipids span a discontinuous bilayer, with their hydrophobic tails pointing towards the interior of the membranes and their hydrophilic head groups in contact with the water phase outside the membrane. In this bilayer integral proteins are embedded, occasionally crossing the total lipid bilayer matrix, while peripheral proteins are bound exterior to the bilayer. Dependent on e.g. temperature and water content\textsuperscript{3}, the more or less extended hydrocarbon chains of the lipids tilt away from the perpendicular to the plane of the membrane, thus changing the ratio of the cross sectional areas of head group and chain region\textsuperscript{4}. In this way a modification in density of the membrane can be reached, comparable to the melting phenomena of classical chemical compounds, e.g. from a fluid liquid-crystalline to a solid gel-like phase. This melting can, even for pure lipids, not be described as a thermodynamic first-order phase transition, since the transition is certainly not discontinuous, as shown, for example, by measurements of volume changes\textsuperscript{5}. One can imagine a gel-like phase in which an appreciable lateral diffusion of the lipid molecules exists, while on increasing temperature this lateral diffusion will be accelerated throughout a phase-transition region to the fluid liquid-crystalline phase. The width of this phase-transition region will be increased when mixing different lipids with each other, with proteins or with other membrane constituents\textsuperscript{5}. In natural membranes local gel-like domains\textsuperscript{6} exist over a large temperature range in liquid-crystalline matrices and vice-versa. These domains, often called clusters, develop in a continuing process of ordering and successive relaxation to a disordered state. They consist of mainly lipids, mainly proteins or a mixture of both, but always can be described as a region of different density compared to the surrounding matrix. Diffusion over longer distances sometimes will be opposed in biological membranes by a cytoskeletal system localizing essential proteins in a well-defined region and by
a number of multivalent ligands that can induce aggregation of proteins into clusters, patches or caps\textsuperscript{7} in an alternative way as mentioned above. Here proteins reside in a defined region near to each other, but are not necessarily located in one and the same cluster in the sense of same degree of order or same density. When the word cluster is used throughout this thesis, a small region of one aggregational state in a matrix of another will be ment, the first description of clusters given above.


There is a lot of controversy concerning the role of lipids in a membrane. Some authors state that lipids are the insulating constituents of the membrane, separating different cellular compartments\textsuperscript{8}. They form the structural support of membrane proteins, thus maintaining a constant spatial relationship between them. In this view ion transport across

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{lipid_composition}
\caption{Lipid composition by weight of different subcellular membranes of rat liver; PL = phospholipid; C = cholesterol; G = glycolipid; unlabelled = mono-, di-, and triacylglycerols, free fatty acids and stearyl esters.}
\end{figure}
membranes is a property of integral proteins, that are influenced by external factors like metal-ions, protons, potential field etc. If one considers however, that lipid composition of animal membranes vary both with their tissue source and intracellular location (see Figure I.2 and I.3), the question is raised whether the lipids could play a role themselves. So it has been suggested that the level of free fatty acids, that varies with the functional state of the membrane, may be involved in changes in membrane permeability. Until now, little attention has been paid to the varying amounts of phospholipids in the membranes, in particular to the transition of the four co-ordinated to the five co-ordinated state of the phosphate group, bound on its crucial position between the polar headgroup and hydrophobic hydrocarbon region, in relation to the phenomenon of ion transport. Recent studies on a number of model compounds for biological reactives suggest a general principle in passing biological information from ionic polar regions to hydrocarbon zones of natural molecules via a five co-ordinated \((P(V))\) trigonal bipyramidal (TBP) intermediate (see Figure I.3. Phospholipid composition by weight of different subcellular membranes of rat liver; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PS = phosphatidylserine; S = sphingomyelin.)
Chapter I.3. and I.4.). A classical four co-ordinate (P(IV))
tetragonal intermediate, possesses four ligands that are
arranged spherically around the phosphorus atom, i.e. the P-L
bond lengths and the L-P-L bond angles are equal. When a five
co-ordinated TBP intermediate is formed, a structural
inequivalence between two types of ligands (the equatorial
and the axial ones) is introduced, since five ligands can not
be spherically arranged around one atom (see Figure I.4.).

![Diagram of T and TBP](image)

*Figure I.4. The four co-ordinated tetraeder (T) and the five co-ordinated trigonal bipyramidal (TBP) configuration of phosphorus.*

The axial ligands (an incoming group e.g. water and the group
through which the phosphate is bound to the main chain of the
biomolecule) are more electron withdrawing groups (see
Chapter I.3.) than the equatorial ones, thus inducing an
electron flux into the axis of the TBP. If the group in the
axis consists of the O-C-C-O-sequences often encountered in
biomolecules, this extra negative charge on the axial oxygen
(031 in Figure I.5. in the case of lipids), will result in a
repulsion of the other oxygen (021 in Figure I.5.). In this
way a conformational change in the phospholipid headgroup
will be transferred in a re-orientation of the hydrophobic
region of the lipid molecule. As will be explained in Chapter
II, this re-orientation can induce cluster formation, which
in turn may influence integral membrane proteins as ion
channels, thus triggering them to open or to close. To get
experimental support for this model, a number of vesicles has
been synthesized with different lipid composition. The
influence of this lipid composition on the ion transport over
Figure I.5. The extra negative charge on the apical position of the five co-ordinated phospholipid intermediate results in repulsion of the oxygen bound via an P-O-C-O-O sequence.

the vesicle cell wall, using one and the same ionophore, is described in Chapter II to investigate several aspects of the theory.

I.3. Developments in organophosphorus chemistry.

In the past few decades, research in organophosphorus chemistry has developed enormously\(^1\). Study of the reactivity of model compounds has greatly enhanced the comprehension of the properties of five co-ordinated phosphorus compounds. So Westheimer's studies\(^1\) on the hydrolysis of five-membered cyclic phosphonates have increased the understanding of the mechanistic aspects of phosphorylation reactions. It was found that the hydrolysis of five-membered ring phosphates as 1 in Figure I.6., proceeds millions of times faster in comparison to acyclic phosphates as 4. This will apply for both ringopening (a) and exocyclic cleavage (b) of 1. In contrast, the cyclic phosphonate 5 gave only very fast ring opening, no exocyclic hydrolysis. Westheimer explained these observations on the
assumption, that the hydrolysis proceeds via a penta co-ordinated intermediate in a trigonal bipyramidal configuration. Before these phenomena can be understood, some properties of five co-ordinated phosphorus compounds should be noted. As already described in chapter I.2. an important aspect of five co-ordination is, that the distribution of the ligands can not be spherically around the central atom, i.e. the ligands are not equivalent\textsuperscript{18}. Two possible structures are favoured, as shown by X-ray analysis\textsuperscript{19-21}: the trigonal bipyramid (TBP) and the square pyramid (SP), shown in Figure I.7. In the TBP there are three equivalent equatorial and two axial bonds, in the SP one axial and four basal bonds. Theoretical considerations based on MO and electrostatic calculations have predicted that the TBP is slightly more stable for acyclic penta co-ordinated phosphorus derivatives\textsuperscript{18}, but the difference is not too large. In

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure16.png}
\caption{Cyclic and acyclic phosphates used in the studies of Westheimer.}
\end{figure}
general an ideal TBP is seldom encountered, mostly a TBP is slightly distorted towards an SP geometry. In the TBP configuration the axial bonds are longer and usually weaker than the equatorial bonds, a picture that can be ascribed by a pd-hybridization for the axial22-24 and a sp2-hybridization for the equatorial bonds. However, the exact role of d-orbitals is still a subject of controversy25-27. Recent publications suggest a remarkable degree of s-character in the axial bonds of some radicals28-30. So the observed differences between axial and equatorial sites in the TBP structure can, in a more differentiated picture, better be described by a substantially higher s-character for equatorial than for axial bonds. In addition, axial sites are preferred by electron withdrawing ligands, whereas electron donating ligands tend to occupy equatorial positions31. This polarity rule has been derived from many experimental data32,33, and is supported by semi-empirical calculations34,35. Furthermore, it has been found that small rings usually span an axial and an equatorial position in the TBP configuration, due to the 90° angle between these two bonds16. This is known as the strain rule. In fact, the presence of rings stabilizes this configuration to such an extent, that most of the known stable phosphoranes contain one or more rings. One of the consequences of the differences in bond strength in a TBP is that leaving groups depart from the axial position16,34. Due to the microscopic reversibility16, incoming nucleophiles also enter in the axis of the TBP34. An aspect of five co-ordination which hampers
differentiation of axial and equatorial bonds in the TBP configuration, is the existence of pseudorotation. Pseudorotation for phosphorus compounds involves that the positions of the ligands are interconverted fast on NMR time-scale\textsuperscript{36-38}. Several types of these 'permutational isomerizations' are known, e.g. the Berry pseudorotation, in which two equatorial and both axial ligands change places\textsuperscript{39} via an intermediate SP configuration (see Figure I.8.).

\begin{figure}[h]
\centering
\includegraphics[width=0.7\textwidth]{figure1.png}
\caption{The Berry pseudo-rotation process.}
\end{figure}

The energy barrier for pseudorotation may be very low, especially if all ligands are identical\textsuperscript{18}. However, if pseudorotations bring electron withdrawing ligands into equatorial positions\textsuperscript{34,40}, or force small rings to span two sites of the same kind\textsuperscript{34}, pseudorotation will be severely hindered. Using the properties described above, the experiments of Westheimer (vide supra) are now readily explained (see Figure I.9.). Initial attack of water on 1 yields intermediate 7. Subsequent proton transfer towards the endocyclic axial oxygen atom leads to formation of 8, resulting in 2 after ring opening (the axial P-O bond is broken). However, if the P(V) intermediate 7 undergoes ligand reorganization, 9 is formed. Upon leaving of the axial protonated methoxy group, 3 is generated. The very fast rate of both processes is explained by the fact that cyclic, four co-ordinated phosphorus compounds are more strained than their acyclic analogues, whereas cyclic phosphorane intermediates such as 7 or 9 are stabilized with respect to acyclic phophoranes. These factors lower the activation
enthalphy for hydrolysis of cyclic compounds substantially. Phosphonates as 5 can (see Figure I.6.), after attack of water and proton transfer to the axial oxygen, isomerize to either an intermediate with the ring carbon in an axial position (normally not occupied by electron donating groups), or to an intermediate with a di-equatorial five-membered ring, increasing the ring strain. These processes are energetically unfavourable and cannot compete with ring opening, so no exocyclic hydrolysis is found with the phosphonate.

More recently, many other stereochemical and kinetic data in phosphorylation reactions and in group transfer reactions have been rationalized by invoking phosphorane intermediates, including the group transfer reactions in tricyclic 'caged' phosphatranes by Van Aken et al. \(^{42,43}\).
1.4. Transfer of conformational changes in a phosphate group to the hydrophobic part of organic molecules.

In the phosphorylation reactions of Westheimer, substitution was achieved at phosphorus, whereby both incoming and leaving groups entered and departed via the axis of an P(V) TBP. In biochemistry a lot of phosphate groups are temporarily activated without breaking the bonds with the rest of the biomolecule. In this case incoming and leaving group are the same molecule, e.g. water. Dependent on the life time of the P(V) TBP intermediate the activated system will be able to relax to a lower energy state. Another configuration will be occupied, that is better able to accommodate the new charge distribution over the ligands around phosphorus. Usually this will happen by turning away one electronegative part of the molecule from the other. Theoretical verification of charge

| ligands of a tetrahydrofurfuryl model system when going from a four to a five co-ordinated intermediate; CND0-2 optimized results. |
enhancement on apical ligands in a P(V) TBP model compound for DNA was recently published by van Lier et al.\textsuperscript{11}. The net atomic charge on various atoms in the tetrahydrofurfuryl model system and its P(V) TBP counterpart are given in Figure I.10. Experimental evidence for the rotation, resulting from this charge enhancement, was very recently given by Koole et al.\textsuperscript{12}. They synthesized a number of four and five co-ordinated mutually resembling phosphorus model compounds and found a significantly greater population of the gauche(-) conformation for axially situated tetrahydrofurfuryls around the C₄'⁻C₅' bond in the 5' P(V) TBP tetrahydrofurfuryls with respect to their related P(IV) compounds. In Figure I.11., some of the model compounds used are given\textsuperscript{12}.

Figure I.11. Four and five co-ordinated model compounds with different ligands substituted on the phosphate group and in the tetrahydrofurfuryl ring.

The Newman projections of the rotamers around the C₄'⁻C₅' bond are given in Figure I.12. The rotamer populations x(g⁺), x(g⁻) and x(g⁻') could be determined from the time-averaged coupling-constants J₄'H₅' and J₄'H₅"\textsuperscript{12}. In the P(IV) compounds Os' and Y are oriented cis to each other (the gauche(+) or the gauche(t) rotamer) in the case Y = O, due to the gauche effect\textsuperscript{44}. This effect is defined\textsuperscript{45} as the "tendency to adopt that structure which has the maximum
number of gauche interactions between the adjacent electron pairs and/or polar bonds and originates from bond-antibond interactions. The only compound that differs substantially possesses a CH\textsubscript{2} group on the Y position (see Figure I.11.) and the C\textsubscript{4'}-C\textsubscript{5'} rotamer distribution is consequently not dominated by the gauche effect. In the P(V) compounds O\textsubscript{5'} and O\textsubscript{1'} are orientated more trans to each other, i.e. the g\textsuperscript{-} population is enhanced, due to the repulsion of two more negatively charged oxygens. Only the compounds with Y = CH\textsubscript{2} show no difference in population with the four co-ordinated intermediate, which is clearly the consequence of the P-O-C-C-C sequence present in the molecule, instead of the P-O-C-C-O sequence, that is responsible for repulsion.
References and notes.

7. In reference 1, p 119.
8. In reference 1, p 12.
10. In reference 1, p 86.
13. For up-to-date reviews on the subject, see the series 'Organophosphorus Chemistry' (Specialist Periodical Reports), S. Trippett, ed., The Chemical Society, London.
II. Sodium ion transport across biological membranes.

II.1. Theory.

II.1.1. Cluster formation in biological membranes induced via a phospholipid P(V) TBP intermediate.

In Chapter I the principle of induction of an electron flux into the axis of a five co-ordinated phosphorus (P(V)) trigonal bipyramidal (TBP) intermediate was discussed. In those model compounds, the extra negative charge on the axial oxygen ($O_5'$) in the P(V) TBP intermediate resulted in repulsion of another oxygen ($O_1'$), bound via an O-C-C-O sequence to the phosphate group, and located in a tetrahydrofurfuryl ring (see Figure II.1(a)).

![Diagram](image)

*Figure II.1. Repulsion between the two oxygens in a P-O-C-C-O sequence as a consequence of the transition from a four co-ordinated to a five co-ordinated intermediate in (a) a tetrahydrofurfurylphosphate and (b) a phospholipid.*
In this Chapter, an attempt will be made to show that the same process can occur in phospholipids, and that this process could be the 'trigger' to activate proteins, such as ion channels, embedded in a lipid bilayer matrix via uptake in clusters. In Figure II.1(b) one can distinguish the same O-C-C-O sequence, bound at the axial position of a phosphate group, as discussed for the model compounds in Chapter I. Repulsion between the two oxygens O$_2$ and O$_3$ of the P-O-C-C-O sequence will cause a shift of the sn-2 chain in the direction perpendicular to the bilayer surface (see Figure II.1.(b)). However, the model compounds as (a) in Figure II.1. are monomers, dissolved in organic solvents, and thus able to re-orientate freely in solution. The phospholipids, on the contrary, are built in in the lipid bilayer, with their long hydrocarbon chains interacting via 'van der Waals' interactions with the neighbouring chains. So the shift of the hydrocarbon chains along each other will normally take a high energy barrier to overcome. A plausible adaptation of the bilayer by which this process can be aided, is accompanied by a change in the angle of tilt of the hydrocarbon chains to the bilayer normal. Phase diagrams of phospholipids show, dependent on temperature and percentage water or different lipid, several one and two phase regions, in which among others the angle of tilt to the bilayer normal varies. In a special temperature interval, the phase transition region, ranging from the main phase transition temperature down to a temperature around the pretransition, small domains of different density (and thus different angle of tilt) occur next to each other. Such clusters are reported for mixtures of phosphatidylcholines (PC) with phosphatidylethanolamines (PE), cholesterol or proteins. The co-operative change in the angle of tilt of all the lipid molecules in the same cluster, can minimize the energy barrier that has to be overcome. Although there is controversy about the exact nature of the pretransition, a continuous change in the angle of tilt is always included in the description. Some authors conclude that the angle of tilt will change from tilted at
the pretransition temperature to parallel to the bilayer normal at the main phase transition. Others believe the angle of tilt reaches a local minimum at the pretransition, accompanied by a transition from a tilted conformation, via a tilted and rippled two dimensional structure to a one dimensional structure with the chains perpendicular to the bilayer surface. Experimental evidence for a variation in angle of tilt of the hydrocarbon chains accompanying hydrocarbon chain shift was given by Blume and Chen. Comparison of $^{13}$C and $^2$H NMR spectra of phospholipids, labelled respectively with $^{13}$C at the sn-2 carbonyl group and with $^2$H at the 4-position of the same sn-2 chain, suggests that a conformational change of the carbonyl group precedes chain melting on increasing temperature. This could be an indication of constantly developing P(V) TBP intermediates, meeting below the pretransition.

<table>
<thead>
<tr>
<th>lipid</th>
<th>main transition temperature</th>
<th>pretransition temperature</th>
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<tbody>
<tr>
<td>MMPC</td>
<td>23.6</td>
<td>14.4</td>
</tr>
<tr>
<td>MPPC(a)</td>
<td>35.1</td>
<td>22.8</td>
</tr>
<tr>
<td>MSPC(b)</td>
<td>38.6</td>
<td>-</td>
</tr>
<tr>
<td>PMPC</td>
<td>27.3</td>
<td>10.8</td>
</tr>
<tr>
<td>PPPC</td>
<td>41.1</td>
<td>34.8</td>
</tr>
<tr>
<td>PSPC</td>
<td>49.0</td>
<td>39.9</td>
</tr>
<tr>
<td>SMPC</td>
<td>29.4</td>
<td>20.0</td>
</tr>
<tr>
<td>SPPC</td>
<td>43.9</td>
<td>30.8</td>
</tr>
<tr>
<td>SSPC</td>
<td>54.2</td>
<td>50.4</td>
</tr>
</tbody>
</table>

(a) MPPC = a myristoyl chain (M) bound at the sn-1 position and a palmitoyl chain (P) bound at the sn-2 position of the phosphatidylcholine (PC) glycerol backbone.

(b) S = a stearoyl chain.

Table II.1. Main transition and pretransition temperature as a function of the chain length of the chain at the sn-1 and the sn-2 position of phosphatidylcholine.
sition temperature\textsuperscript{3} an energy barrier too high to transmit
the repulsion between the two oxygens of the O-C-C-O sequence
in a shift of the sn-2 chain. The activation around the pre­
transition temperature is high enough, however, to induce a
conformational change at the carbonyl-group next to O\textsubscript{21}.
At higher temperatures an ever greater part of one and the
same sn-2 chain and/or an ever greater part of the total
number of sn-2 chains will be activated, untill at the main
phase transition all the chains are oriented perpendicular to
the membrane surface. Moreover, the pretransition behaviour
shows strong dependence on composition\textsuperscript{10}. PCs with myristoyl
(C\textsubscript{14}), palmitoyl (C\textsubscript{16}) and stearoyl chains (C\textsubscript{18}) at the sn-1
and/or sn-2 position meet a higher energy barrier to melt if
the sn-2 chain is longer (see Table II.1). Another environ­
mental constraint is met in the headgroup region (see Figure
II.2.).

![Diagram]

Figure II.2. Ringformation in the phosphatidyicholine headgroup upon
formation of a five co-ordinated intermediate.

In the headgroup, accommodation of a fifth ligand to form a
five co-ordinated intermediate will cause re-orientation of
the ligands around phosphorus. A lot of phospholipid molecules contain zwitterionic headgroups (see Figure II.3.), that are arranged with alternating charge in the bilayer\textsuperscript{11}. This model of intermolecular interaction between e.g. the N-methyl protons of one PC molecule and the phosphate of a neighbouring PC molecule, however, still allows for considerable freedom of movement about the various bonds in the headgroup\textsuperscript{11}.

\begin{center}
\begin{tabular}{ccc}
\includegraphics[width=0.3\textwidth]{phosphatidyl-choline} & \includegraphics[width=0.3\textwidth]{sphingomyelin} & \includegraphics[width=0.3\textwidth]{phosphatidyl-ethanolamine}
\end{tabular}
\end{center}

phosphatidyl- sphingomyelin phosphatidyl- phosphatidyl-choline ethanolamine serine

*Figure II.3. Phospholipids with a zwitterionic headgroup.*

Thus formation of a five co-ordinated phosphorus intermediate must be accompanied by re-organization of the charge in the total bilayer. This can occur, for example, by intramolecular compensation of the charge\textsuperscript{12}, thus creating a more or less neutral molecule, or, at a physiological level, by de- and adsorbtion of mono- and di-valent cations\textsuperscript{13,14}. Intramolecular compensation of charge can be established by pseudo-ringformation, in which positively and negatively charged groups are brought close to each other\textsuperscript{12} (see Figure II.4.). Here, another aspect of the pretransition behaviour is met, the relative cross-sectional areas of headgroup and chain region. In molecules as PC, the headgroup in 'stretched' P(IV) conformation, will occupy a greater
excluded cross-sectional area than the lipid hydrocarbon chains. The chains adopt a tilted conformation to fill in a potential void in the hydrocarbon chain region. Above the pretransition temperature, an increasing number of chains orient more perpendicular to the membrane surface, so that the excluded cross-sectional area of the headgroup must have been diminished. This is confirmed by the observation that PE, N-methyl and N,N-dimethyl PE do not exhibit such pretransition behaviour, since the cross-sectional areas of their headgroups are smaller. The effective cross-sectional area of the PC headgroup can be diminished further, after di-equatorial pseudo-ring formation and pass down of the charge, by pseudo-rotation, through which the pseudo six-membered ring will be orientated temporarily axial-equatorial. This pseudorotated structure will, at the same time, prevent electron back donation to the fifth ligand, e.g. water, and return of the intermediate to the four co-ordinated state. De- and adsorption of mono- and divalent cations can complete the picture sketched above. During the physiological process of the excitation of an axon, for example, momentary desorption of Ca2+ ions from the outer monolayer of the membrane is reported upon activation of the axon, followed by adsorption of monovalent ions as Na+. This decrease in positive surface-bound charge of cations can diminish repulsive forces, intended to keep the headgroup 'stretched' in the unactivated axon.

Finally, again in the example of the excitation of the axon, the potential at rest is negative inside the axon, pulling the positively charged end of the stretched headgroup -N(CH3)3+ of the outer monolayer phospholipids into the membranes. During activation of the axon, a positive potential inside will push the positive charged end of the headgroup outwards, enabling the headgroup to re-orientate. Thus, a set of environmental physiological conditions is realized, enabling a P(V) intermediate to develop and to pass the information, stored in its renewed charge-distribution, down to the hydrocarbon region, for the case of the motionally
restricted phospholipid molecules. On a molecular level this process can be summarized as follows (see Figure II.4.).

Ca\(^{2+}\) ions desorb, and are replaced by Na\(^{+}\) ions. The internal potential is changing from negative to positive. As a consequence the headgroup is no longer forced in an extended and inward pulled conformation and gets the opportunity to re-orientate. The always existing P(IV) \(\rightleftharpoons\) P(V) equilibrium, under 'resting' conditions laying at the side of the P(IV)

![Image](image-url)

*Figure II.4. The changes in the physiological conditions accompanying the transition of a four to a five co-ordinated intermediate.*

compounds, will be shifted in the direction of the P(V) intermediate, since this conformation now is stabilized by the formation of a di-equatorial\(^{16}\) pseudo-six membered ring. The positively charged nitrogen of the choline headgroup shields the negative charge of the formerly double bonded oxygen, thus polarizing the P=O bond, by which the electrophilicity of the phosphorus atom will be increased\(^{18}\). This process will be promoted by nucleophilic attack of e.g. a water molecule\(^{19}\), normally present in excess in the headgroup
layer of the membrane, thus generating the P(V) TBP intermediate. The decrease in cross-sectional area of the head-group due to ring formation, will cause decrease in the angle of tilt of the hydrocarbon chains. The increased electron density on the axial oxygen of the phosphate group will induce repulsion of O_2, bound via an O-C-C-O sequence to the phosphate group. This process will aid, or maybe it is the main cause, of a further decrease in the angle of tilt of the hydrocarbon chains, through a 'shift' of the hydrocarbon chains along each other. The difference in effective chain length\textsuperscript{10, 20-22} is diminished. Co-operative change in the angle of tilt of a number of phospholipid molecules is needed to maximize the 'van der Waals' interactions between neighbouring acyl chains. This will lead at a macromolecular level to formation of a cluster with an average angle of tilt differing from the surrounding matrix. The relaxation time of such a cluster is appreciably greater than for other characteristic movements of the molecule\textsuperscript{23}. So the short-living P(V) TBP intermediate initiates the formation of a cluster with a much longer life-time, through which a time scale can be reached at which physiological processes can take place\textsuperscript{23}. Although the physiological conditions of the above process are borrowed from the excitation of an axon, one can imagine the same conditions for other membranes. Over most membranes an ion-gradient is maintained by ion pumps, so a potential exists over most membranes. Divalent ions as Ca\textsuperscript{2+} and Mg\textsuperscript{2+} are bound to most membrane surfaces to an extent dependent on the physical state of the lipids\textsuperscript{23}. Mostly they are bound more strongly than monovalent ions such as Na\textsuperscript{+} and K\textsuperscript{+}, that are present in all intra and extra-cellular spaces. The local circumstances may change, a P(V) TBP intermediate can be built up under several sets of conditions.
II.1.2. Activation of membrane proteins by uptake in phospholipid clusters

A break in the Arrhenius plot of ATP-ase activity versus the reciprocal temperature has been reported in dioleoyllecithin substituted ATP-ase, when reaching the temperature of cluster formation. A transition in the temperature dependence of Ca$^{2+}$ accumulation in Sarcoplasmic reticulum membranes is attributed to a change in entropy of activation rather than to the free energy of activation. Results that are consistent with an order-disorder transition involving the lipid alkyl chains. Computer simulation studies concerning phase separation in lipid bilayers containing integral proteins, show a system that separates into an essentially pure lipid phase and a protein-rich phase containing melted lipids between $T_k$ and $T_C$. Here $T_k$ is the melting point of clusters, and $T_C$ is the (main) melting point of lipids. Addition of oleic acid to a lipid deficient membrane produces a fluid membrane structure, which is most likely an essential requirement for the reconstitution of the calcium dependent ATP-ase activity. Addition of stearic acid, on the contrary, has no activating effect on the calcium dependent ATP-ase and creates a gel-like lipid structure. From the above-mentioned and other articles, it becomes clear that a certain fluidity is essential for the activation of membrane proteins, and that this fluidity is reached in a temperature range in which cluster formation appears.

An often encountered objection against the relation between cluster-formation and protein activation is, that gel state lipids do not appear to be present in most biological membranes. However, this is only partly true. Harrison and Lunt conclude that, although hydrocarbon chains in natural membranes are believed to be generally in a fluid state at physiological temperatures, the presence of sterols and proteins may lead to local variation of the mobility in the membrane. Moreover, the degree of lateral phase separation is believed to be influenced by a number of external factors,
e.g. water content\textsuperscript{31}, proton and cation concentration\textsuperscript{32}, ionic strength\textsuperscript{32} and potential field\textsuperscript{19}. Under influence of the above-mentioned factors, cluster-formation is believed to persist far above the main phase transition temperature of a pure lipid mixture. Also pore-mediated ion transport shows some peculiar characteristics while planar bilayer membranes pass the phase transition region on heating\textsuperscript{33}. Planar bilayers consisting of mixed-chain lipids and modified by pore-forming antibiotics as Gramicidin A, do not show any peculiar effect on T\textsubscript{C}, the main phase transition temperature (29°C). However, at 22-23°C a pronounced maximum in pore-induced conductance is seen. The effects observed are interpreted in terms of lateral phase separation into pure lipid and lipid-antibiotic domains\textsuperscript{33}. Consequently, the polypeptide Gramicidin A is an ideal model for the ion-channel forming proteins, obeying the same temperature dependence of protein activation upon cluster formation. A schematic representation of Gramicidin A\textsuperscript{34} is given in Figure II.5.

Figure II.5. The Gramicidin channel comprises two polypeptide chains in \textbeta-helix form.
On a molecular level, protein activation upon uptake of the protein in a cluster, can be described as follows. An integral protein in a fluid environment (the cluster) could be free to adopt the tertiary structure necessary to function as an ion-channel. A gel-like matrix can displace some special group of the channel-forming protein out of its critical position and/or disturb a protein in helix form. For the sodium ion-channels of myelinated axons a model is given in which four energy barriers in the pore comprise the selectivity filter in the ion-channel. These four barriers consist of dehydration and hydration steps, enabling a partially dehydrated sodium ion to pass a narrow gap besides a strongly co-ordinating carboxylic acid group. Completely hydrated, the sodium ion will not be able to pass the narrow selectivity filter of 3 x 5 Å. A very small displacement of the carboxylic acid group can make the ion-channels impermeable.

![Figure II.6. The four energy barriers comprising the selectivity filter of the ion channel.](image)

Although the correlation between a fluid lipid environment and activation of proteins is suggested by quite a number of authors (vide supra), this is not necessarily a general
principle. Also the reverse process, i.e. protein activation upon uptake in a gel-like cluster, is a process that should not be neglected. In this way a number of different proteins could be activated in succession, if e.g. the potential is constantly changing from negative to positive and a whole scala of states of different rigidity is passed through. Finally, support for the above-mentioned theory is given by the fact, that 2-amido PC, contrary to PC, is found to be inhibitory for integral proteins\textsuperscript{39,40} (see Figure II.7.). The oxygen esterified to C\textsubscript{2} of the glycerol backbone of PC is essential for the transfer of conformational change in the headgroup towards the change of tilt of the hydrocarbon chains. If this oxygen is replaced by the less electronegative nitrogen of 2-amido PC, less repulsion and resulting acyl chain shift will be expected. Moreover, the hydrogen bridge found in X-ray analyses of comparable lipids\textsuperscript{41}, will hinder acyl chain shift and headgroup re-orientation (Figure II.7.).

\textbf{Figure II.7. Hindered repulsion and acyl chain shift in 2-amido phosphatidylcholine.}
II.2. Experiments.

II.2.1. Introduction.

The characteristic feature of the model, described in this Chapter, is that changes in the lipid environment of a protein are the 'trigger' for the protein to be activated (to place some particular functional group just in or just out of the right position). Other authors propose a mechanism in which changes in membrane potential, pH, ionic strength etc., directly influence the channel-forming protein (in the case of ion-transport) to open, a mechanism that developed under the influence of experiments with the ion channel blockers tetrodotoxin\textsuperscript{42} and saxitoxin\textsuperscript{43}.

To get a more decisive answer about this question, the experimental conditions of the investigations described below are chosen so, that only the lipid composition of the membranes varies, leaving the concentration of ion channels, ions, probes and buffers, as well as temperature, as constant as possible. Vesicles are formed with a diameter of approximately 1000 Å, their wall existing of one double layer of lipids. As a reference, vesicles of egg yolk lecithin are chosen, to which 10 to 50\% of synthetic or natural, specific lipid is added, to vary the total lipid composition. Attempts to make vesicles of one well-defined synthetic lipid as reference, failed, since the temperature of formation of the vesicles had to be above the main phase transition temperature, $T_C$. $T_C$ will vary from 24°C for dimyristoyl (C\textsubscript{14}), via 41°C for dipalmitoyl (C\textsubscript{16}) to 55°C for distearoyl (C\textsubscript{18}) phosphatidylcholine\textsuperscript{44}. The last two temperatures where too high to be constantly maintained throughout the whole procedure of synthesis. Since $T_C$ of egg yolk lecithin is around 0°C, the choice of this lipid experimentally gave no problems.

As model for the ion-channel protein, the ionophore Gramicidin A was chosen for a number of reasons, in addition to those mentioned in Chapter II.1.2. Gramicidin A is a pore-former, specific for sodium ions. It is a pore-former and not
a carrier, so it builds up a permanent channel comparable to natural ion-channels, and does not diffuse through the membrane as carriers do. A pore-former also functions below the main phase transition temperature so that the temperature range in which it is active is greater, while the conductance of carriers falls below $T_c$ to the state of bare membrane conductance (not pore mediated)\textsuperscript{33,45}. The spontaneous current fluctuations observed with unmodified planar bilayers near the lipid phase transition temperature, containing a few molecules of Gramicidin A, reminds of the idea of 'cluster-activation' of channels\textsuperscript{46}, i.e. Gramicidin A is activated if it is taken up in such a cluster. Formation of a cluster around a Gramicidin A molecule activates the channel to open. The channel stays open during the life-time of a cluster, that can change randomly, but the conductance reached is always the same, unless the cluster will decay before the maximum conductance is reached.

A Gramicidin A channel is formed by association of two polypeptides at their N-formyl ends. Each chain is folded into a $\beta$ helix, which resembles a rolled-up $\beta$ pleated sheet (see (a) in Figure II.8.).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{gramicidin_channel.png}
\caption{Four possible conformations of the Gramicidin channel.}
\end{figure}

Finally, Gramicidin A incorporates spontaneously in the vesicle wall after addition, since its amino acid sequence is one of the most hydrophobic ones known\textsuperscript{47}.  

38
Figure II.9. $^{23}\text{Na}$ NMR spectra (79.4 MHz) of a dispersion of vesicles of egg yolk phosphatidylcholine plus 10% sphingomyelin (a) at $t=0$ before addition of the probe, (b) at $t=0$ after addition of the probe, (c)-(l) after addition of Gramicidin; $T = 25^\circ\text{C}$. 
Moreover the exterior of the channel consists again of the most hydrophobic parts of the peptide, thus creating an oxygen lined, probably water-filled, central channel. Gramicidin A is added in last resort to a buffered solution with approximately 1000 Å diameter vesicles, around which the NaCl solution is replaced by LiCl by means of ultrafiltration. Thus a sodium ion gradient is formed of about 10^2, the equilibration of which is started at the moment Gramicidin A is added. A shift reagent, Dy[N(CH2CO2)3]2^3- has been used to distinguish between 23Na+ inside and outside the vesicles.

II.2.2. Results.

A homogeneous suspension of 1000 Å diameter, single bilayer phospholipid vesicles is prepared by the procedure of Enoch and Strittmatter. The solubility and nature of the surfactant deoxycholate, together with its concentration (a phospholipid to deoxycholate molar ratio of 2:1 is used), determine the diameter of the resulting vesicles. After formation of the vesicles, the detergent is removed to make the vesicles impermeable to sodium ions, next external NaCl is replaced by LiCl by means of succeeding ultrafiltration steps. After this stage there is 100 mM NaCl inside the vesicles and the aqueous space outside the vesicles is 50 mM in LiCl and < 1 mM in NaCl. The 23Na NMR spectrum (79.4 MHz, Bruker CXP-300) of a dispersion of vesicles consisting of egg yolk lecithin +10% sphingomyelin is given in Figure II.9.a, the single sharp resonance at 1802 Hz ± 2 Hz representing both the Na+ inside and outside the vesicles. Fig. II.9.b shows the spectrum after the outside aqueous space is made 3 mM in triethanolamine dysprosium nitrilotriacetate [HN(CH2CH2OH)3]3Dy[N(CH2CO2)3]2, according to the method of Pike et al. The single resonance of Figure II.9.a is split into two peaks, the smaller one 65 ± 10 Hz upfield from the peak position of Figure II.9.a representing Na+ outside the vesicle and the large one 4 ± 1 Hz downfield representing Na+.
Figure II.10. The time dependence of the relative integrals of the two peaks of Figure II.9, for ten different samples, differing in lipid composition of the vesicle cell-wall.
inside, the absolute magnitude of the shift being dependent on the exact concentration of the probe\textsuperscript{49}. The fraction of the total integral, due to the inside resonance varied from 0.77 to 0.92 for the various samples. The value for the fraction of the total aqueous volume inside the vesicles is calculated, assuming an internal volume of 2960 ml per mole of lipid\textsuperscript{52} and a final lipid concentration of 10 mM, to be 0.05. There could be some error in this ratio because of inaccuracy in the knowledge of the final lipid concentration. However, other studies using the method of Enoch and Strittmatter\textsuperscript{50}, where phosphate analyses was conducted on the final solutions, were in good agreement with the above-mentioned fraction of lipid left after synthesis of the vesicles. Combination of these numbers with 100 mM Na\textsuperscript{+}\textsubscript{in}, yields a Na\textsuperscript{+}\textsubscript{out} concentration of 0.27 mM in the case the fraction of the total integral due to inside resonance at t = 0 is 0.92 or 0.92 mM in the case this fraction is 0.77. Since any leakiness of the vesicles would affect the observed ratio, all starting spectra were obtained between 0.5 and 3 hrs after the last ultrafiltration step, and samples with a fraction due to inside resonance smaller than 0.77 were not used further. E.g. for the sample of egg yolk lecithin with 50\% DSPC it was not possible, even after several attempts, to obtain a sample with a fraction due to internal resonance greater than 0.30. Immediately after the spectrum of sample + probe was obtained, the solution was made 0.04 \mu M in the ionophore Gramicidin\textsuperscript{53-55}. This amounts to ca. 3 Gramicidin channels per vesicle\textsuperscript{49} and induces a rapid efflux of Na\textsuperscript{+} down its concentration gradient, as can be seen in the Figures II.9.c-l. They depict some of the spectra obtained and show the time evolution of the spectrum measured in minutes from the time of addition of Gramicidin, for a sample displaying an intermediate time course in the total series measured (egg yolk + 10\% spingomyelin). The spectra given are power spectra in which the integral is proportional to the square of the number of the sodium nuclei\textsuperscript{56}. The power spectra are taken to cancel the influence of the phase-correction, that will vary
with time during the period the automated measurements are recorded. A plot of the logarithm of ratio \( R \), of the fractional integral (inner/total) at \( t = t \) to the fractional integral at \( t = 0 \), against time is shown in Figure II.10. The time during which the spectra were recorded after addition of Gramicidin varied from 2 to 13 hrs, dependent on how fast the ion transport took place. For 50% DPPC not the total time-course is given, since between 5 and 13 hrs after addition of Gramicidin the slope of the plot was identical to that of the part shown between 0 and 5 hrs.

II.2.3. Discussion.

The efflux of \( \text{Na}^+ \) ions shows at least two stages. Directly after addition of Gramicidin a passive one-for-one \( \text{Na}^+ \) for \( \text{Li}^+ \) exchange out of and into the vesicles takes place, both ions moving down their concentration gradients, although the \( \text{Cl}^- \) transport may play a role at this stage (vide infra). This stage will end when the \( \text{Li}^+ \) gradient is dissipated (47.5 mM both inside and outside). Since the \( \text{Na}^+ \) gradient still exists at this point (52.5 mM inside, 3 mM outside, \( R = 0.52 \)), during the second stage the \( \text{Na}^+ \) gradient will be further dissipated at the expense of creating a new \( \text{Li}^+ \) gradient, still through a one-for-one exchange. This stage will end when the ratio of inside concentration to outside concentration has the same value for both \( \text{Na}^+ \) and \( \text{Li}^+ \), so that the same diffusion potential for both ions is reached. Here \( \text{Na}^+ \) in is 10 mM, \( \text{Na}^+ \) out is 5.3 mM, \( \text{Li}^+ \) in is 90 mM and \( \text{Li}^+ \) out is 45.3 mM, the fraction of total \( \text{Na}^+ \) inside is 0.09. True equilibrium will only be obtained after a third stage, namely passive nonfacilitated \( \text{Cl}^- \) transport out of the vesicles. This stage will end when the fraction of total \( \text{Na}^+ \) inside is equal to the analogous volume fraction, 0.05. In these rough calculations osmotic swelling, a possible pH gradient due to permeation of the counter ion of the probe \( \text{HN(CH}_2\text{CH}_2\text{OH})_3^+ \) and the Donnan effect caused by the impermeant
Dy[N(CH₂CO₂)₃]₂⁺ are ignored. The transitions of the curves, shown in Figure II.10, are located between \( R = 0.5 \) and \( R = 0.3 \), the transitions being at lower \( R \) if ion transport is faster. This could be an indication of leakage before Gramicidin is added, although measurements of ten different blancos (after addition of the probe) of the same sample in a time period of half an hour showed no significant decrease of the ratio of \( \text{Na}^+_{\text{in}} \) to \( \text{Na}^+_{\text{out}} \).

The fast process between \( R = 1 \) and \( R = 0.5 \), corresponding to the one-for-one exchange of \( \text{Na}^+ \) and \( \text{Li}^+ \) down to their gradient, is believed to be limited by the Gramicidin induced \( \text{Li}^+ \) transport, which is ca. 1/6th as fast as that of \( \text{Na}^+ \). Thus the slow process, below ca. \( R = 0.5 \), would correspond to the essentially simultaneous occurrence of the second and third stages (vide supra) implying that they have very similar permeability constants. This is supported by measurements of permeability coefficients of passive nonfacilitated transport of \( \text{Cl}^- \). So both stages before and after \( R = 0.5 \), are involved in sodium ion transport.

As can be seen in Figure II.10, the relative sequence in velocity of ion transport for the various samples is not interchanged when passing \( R = 0.5 \).

The reference sample, phosphatidylcholine from egg yolk exists of predominantly C₁₆ (34.3%) and C₁₈ chains (59.8% C₁₈:0, C₁₈:1 and C₁₈:2), as can be seen in Table II.2. Ion transport over a vesicle cell wall consisting of egg yolk phosphatidylcholine is relatively fast, compared to most of the other samples (2-10 in Figure II.10.). Clearly, the relative quantities of different chain lengths and saturation is such, that the rigidity of the matrix is ideal to accommodate for the chain shift that results from the formation of a five co-ordinated phosphorus intermediate. Increasing the percentage of synthetic saturated chains (samples 3-6), the matrix will adopt a more gel-like character, in which the packing of the optimally ordered chains is tighter, so the chain shift is more difficult. When increasing the chain lengths of the saturated chains, ion transport
becomes slower, although the difference between the sample with 10% DSPC and with 10% DPPC is too small, compared to the deviation in slope due to measuring faults, to be called significant. Addition of more (50%) of the same saturated chains again gives slower ion transport, in which the slopes of the plots of 50% DMPC and 50% DPPC are indistinguishable. Addition of 10% of sphingomyelin from egg yolk, containing primarily saturated palmitic acid chains at the sn-2 position results in ion transport that is slower than that of the reference sample of phosphatidylcholine from egg yolk (1), but faster than that of the samples 3-6. The slower ion transport of sample 2 compared to sample 1 could stem from the restricted repulsion of the sn-2 nitrogen (see Figure II.11.) through which the sn-1 chain is bound to the glycerol backbone. The significantly faster ion transport compared to the samples 3-6 can be explained by the fact that the sn-1 chain is significantly shorter (two atoms less than in DPPC, viz. -CH=CH-(CH₂)₁₂-CH₃ directly bound to the glycerol backbone, and the appearance of a double bond), thus creating a molecule comparable to MPPC (see Table II.1.). Moreover, addition of a clearly different lipid will enhance the heterogeneity of the matrix, making the matrix more fluid, and enabling lipids other than sphingomyelin to translate a five co-ordinated phosphorus intermediate more easily in a hydrocarbon chain shift.

Samples 8, 9 and 10 contain additions with primarily C₁₆ or C₁₈:0 to C₁₈:2 (see Table II.2.) as lipid alkyl chains, thus changing the overall fluidity of the matrix not too much, compared to 1, on additions of 10%. Egg yolk phosphatidylcholine with 10% phosphatidylserine shows considerably faster ion transport compared to the reference sample. The availability of two ligands within the serine, one as fifth ligand to build up a five co-ordinated intermediate and one to polarize the original P = 0 bond, could cause an appreciably increased life-time of the five co-ordinated intermediate, which explains the fast ion transport observed (see Figure II.12.).
<table>
<thead>
<tr>
<th>Chain length and saturation</th>
<th>Phosphatidylcholine S-0756</th>
<th>Phosphatidylsphingomyelin P-6013</th>
<th>Phosphatidylethanolamine P-4513</th>
<th>Phosphatidylinositol P-5766</th>
<th>Phosphatidylserine P-8518</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0</td>
<td>0.1%</td>
<td>0.6%</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C16:0</td>
<td>34.3%</td>
<td>86.2%</td>
<td>23.6%</td>
<td>32.8%</td>
<td>-</td>
</tr>
<tr>
<td>C18:0</td>
<td>12.0%</td>
<td>6.3%</td>
<td>3.3%</td>
<td>6.6%</td>
<td>38.5%</td>
</tr>
<tr>
<td>C18:1</td>
<td>31.4%</td>
<td>-</td>
<td>8.0%</td>
<td>6.4%</td>
<td>26.9%</td>
</tr>
<tr>
<td>C18:2</td>
<td>16.6%</td>
<td>-</td>
<td>54.0%</td>
<td>48.0%</td>
<td>-</td>
</tr>
<tr>
<td>C18:3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5.6%</td>
</tr>
<tr>
<td>C22:6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>11.6%</td>
</tr>
</tbody>
</table>

Table II.2. Chain length and chain saturation in natural lipid extracts from Sigma.
Figure II.11. Compared to phosphatidylcholine (a), in sphingomyelin (b) acyl chain shift following from the formation of a five co-ordinated intermediate, is blocked.
Figure II.12. Very fast build-up of a five co-ordinated intermediate in the phosphatidylserine headgroup, due to twofold stabilization.

Phosphatidylinositol possesses a large ligand in the phospholipid headgroup, the diameter of which can hardly be changed by the transition from the four to the five co-ordinated intermediate. A possibly formed five co-ordinated intermediate will not be stabilized by intramolecular ring formation so its life-time will not be long enough to induce cluster formation. Phosphatidylethanolamine already possesses a very small headgroup in stretched conformation (see II.1.1.), so intramolecular ring-formation will not be able to influence the angle of tilt of the hydrocarbon chains. Both samples 9 and 10 display a more or less expected rate of ion transport, somewhat slower than that of phosphatidylcholine (sample 1). Addition of these lipids hardly influences the overall lipid fluidity (the chains display about the same degree of saturation) and will not be able to induce cluster formation.
Figure II.13. (a) Formation of a five co-ordinated intermediate will not be able to influence hydrocarbon chain tilt in phosphatidylethanolamine. (b) A five co-ordinated phosphatidylinositol intermediate will not be stabilized by intramolecular ring formation.
via a P(V) TBP intermediate. So the number of phospholipids that can induce cluster formation (the phosphatidylcholines) is simply reduced with ± 10%.

II.2.4. Conclusion.

The measurements of ion-fluxes through Gramicidin channels over the walls of phospholipid vesicles, enabled us to establish ion transport of different rates, if all the experimental conditions were left constant except the lipid composition. This can be considered as a support for the model, indicating lipid mediated influence of membrane proteins and not just direct influence of proteins by external factors. To test several aspects of the model, in which the transition of a four to a five co-ordinated phospholipid intermediate is the trigger to cluster formation and protein activation, phospholipids with various headgroups and hydrocarbon chains were added to a reference lipid. The results can be considered as a support of the proposed model.

II.2.5. Experimental.

\(\Rightarrow\) Preparation of vesicles.

The vesicles are prepared according to method II of Enoch and Strittmatter\textsuperscript{50}, using the same concentrations noted there. Subsequently, nine ultrafiltration steps (Millipore, Immersible CX-30) with a concentration of 10 to 1 ml in every step, are necessary to reach a fractional integral of internal resonance relative to internal + outside resonance of approx. 0.90. This is an indication that Na\textsuperscript{+} is strongly bound to the external phospholipidmonolayer and that a high concentration ratio between the external phospholipid monolayer and the external solution is needed for the Na\textsuperscript{+} ions to desorb. After the last concentrating step to 1 ml buffered
vesicle solution 1 ml D_2O is added to get the internal reference for the NMR measurements.

➤ Preparation of the probe.

The probe is synthesized according to the double heterogeneous reaction:\textsuperscript{59}-\textsuperscript{63}:

\[ \frac{1}{2} \text{Dy}_2\text{O}_3 + 3 \text{H}_3\text{NTA} + 3 \text{TEA} \rightleftharpoons 3 \text{HTBA}^{+} + \text{Dy(NTA)_2}^{3-} + \frac{3}{2} \text{H}_2\text{O} \]

of 0.746 g Dy_2O_3 (dysprosium oxide, Sigma No. D-0381) and 1.530 g H_3NTA (nitrilotriacetate, Sigma No. N-9877) in 25 ml water with 1.60 ml TEA (triethanolamine, Brocades, s.d. 1.12 - 1.13) to produce 0.16 M of shift reagent\textsuperscript{59}. The pH must never be allowed to rise above 6 or 7 (even transiently) during early stages, lest Dy^{3+} hydrolysis and precipitation becomes a problem. So at first 1.0 ml TEA is added to a stirred suspension of Dy_2O_3 and H_3NTA at a temperature of 40°C over a time-period of two hours. After heating the solution becomes clear at 73°C, the pH is 3.5 at that moment. After cooling to room temperature the solution is cloudy again after standing over one night. Heating to 70°C and addition of the last 0.6 ml TEA will give a clear solution again of pH = 4.5, that stays clear after cooling. Part of this stock solution is brought to pH = 7 with LiOH just before addition of the probe to the vesicle suspension, to keep the pH of that solution as constant as possible and diluted 1:1 with D_2O to obtain a final concentration of 0.065 M of the probe. From this solution 90 μl is added to 2 ml of vesicle suspension during the measurements.

➤ Gramicidin solution.

2.3 mg Gramicidin no. G-5002 (Sigma) per 100 ml methanol gives a stock solution of 1.23 x 10^{-5} M. 6 μl of this solution is added to 2 ml vesicles suspension in 50% D_2O / 50% H_2O. The concentration of the stock solution is made such
that only very little methanol is added to the sample, since methanol is an anesthetic that perturbs the bilayers strongly\textsuperscript{64}.

\begin{itemize}
\item NMR measurements.
\end{itemize}

The \textsuperscript{23}Na NMR (79.4 MHz) spectra are measured on a 300 MHz Bruker CXP-300 spectrometer. For each measurement 256 free-induction decays (FID) are accumulated in 128 s. For each series an average of 8 blanks at \( t = 0 \) (after addition of the probe) is taken as reference \( \text{Na}^+_{\text{in}}/\text{Na}^+_{\text{total}}, t=0 \). After addition of Gramicidin A, the spectra are taken automatically with a computer program, taking successively 30 measurements every 2 minutes, 10 every 4 minutes, 10 every 6 minutes, 10 every 16 minutes and 15 every 31 minutes. If the ratio \( \text{Na}^+_{\text{in}}, t=t/\text{Na}^+_{\text{in}}, t=0 \) falls below \( \pm 0.3 \) the recording is stopped because of too small signal/noise ratio for \( \text{Na}^+_{\text{in}}, t=0 \). Most series are stopped too if \( t \approx 3 \) hours.

As internal lock D\textsubscript{2}O is used, as described by Pike et al\textsuperscript{50}. However, measurements of 2 mM NaCl in H\textsubscript{2}O or H\textsubscript{2}O/D\textsubscript{2}O, with acetone or D\textsubscript{2}O as an external lock, show that the Dys-reagent is a D-shift reagent as well. Since the shift with D\textsubscript{2}O as lock is appreciably greater than without, the choice for D\textsubscript{2}O is maintained.
References and notes.

34. Yu.A. Ovchinnikov, Biomembranes, 8th FEBS meeting, 1972, 279-306.
43. Ibid, p. 1015, 1016.
58. Information provided by Sigma.
Quantum chemical calculations on the stereochemistry of coenzyme B$_{12}$-dependent carbon-skeleton rearrangements.
III. The formation of a carbanionic intermediate in the carbon-skeleton rearrangement step.

III.1 Structure and function of coenzyme B₁₂.

Vitamin B₁₂ is an essential nutritional element for the liver to cure a special form of anaemia, a disease which is characterized by a disturbed ripening and accelerated degradation of the erythrocytes. Vitamin B₁₂ itself is cyano-cobalamin, whose structure was determined by Hodgkin et al., using X-ray crystallography (see Figure III.1.). This structure is composed of two principal parts, the highly substituted, reduced, porphyrin-like corrin ring and the nucleotide, which, unlike those obtained from nucleic acids, contains an α-glycosidic linkage. The corrin ring contains tervalent cobalt chelated to the four nitrogen atoms of this ring, to a nitrogen atom of the 5,6-dimethylbenzimidazole ring and to a cyanide ion, which is an artifact of the isolation procedure. The entire structure, except for the cyanide, is termed cobalamin. In addition to cyanide, hydroxide, water and nitrous acid can be bound to the 6'-position. Vitamin B₁₂ itself is not active as a coenzyme for any known enzymatic reaction, but there exist two coenzymatically active derivatives, 5'-deoxyadenosylcobalamin and aquocobalamin.

\[
\begin{align*}
R'' & \quad \text{H} \\
R' - C - C - R''' \\
\text{H} & \quad \text{H}
\end{align*}
\]

*Figure III.2. [1,2] - Shift of a hydrogen atom and a group R.*

The last one is involved in three biochemical processes, the synthesis of methionine, methane formation and acetate synthesis. Aquocobalamin falls out of the scope of this
Figure III.1. Structure of vitamin B$_{12}$ as determined by X-ray diffraction studies.
thesis. Of the known enzymatic reactions which require 5'-deoxyadenosylcobalamin as cofactor, all but one (ribonucleotide reductase) involve an intramolecular [1,2]-shift of hydrogen coupled with a [1,2]-shift of some other group, as shown in Figure III.2. The eleven rearrangement reactions known until now are divided in three groups, the carbon-skeleton rearrangements, the hydroxyl and the amine migrations, according to the bond that is broken during the reaction: a C-C, C-O or C-N bond. In this study special attention is given to the carbon-skeleton rearrangements, i.e. the isomerization of L-methylmalonyl-Coenzyme A to succinyl-Coenzyme A, threo-β-methylene glutarate to L-glutamate and β-methylitaconate to α-methyl eneglutarate, where hydrogens for sake of clarity deuterons

![Diagram of carbon-skeleton rearrangements](image)

with $R =$

- methyl-
malonyl-Co A
- methyl-
aspartate
- methyl-
itaconate

Figure III.3. The three carbon-skeleton rearrangements dependent on coenzyme $B_{12}$. 
are used in Figure III.3.) and a carbon-centered group R migrate in an intramolecular [1,2]-shift. Under enzymatic conditions the hydrogen (deuteron in Figure III.3.) is transferred via the 5'-methylene group of coenzyme B$_{12}$$^{5-8}$ and migrates in methylmalonyl-Co A with retention of configuration$^{9-11}$ (i.e. the incoming hydrogen and the leaving group R occupy the same position). The migration in methylaspartate occurs with inversion of configuration$^{12,13}$, while the stereochemistry of the methylitaconate isomerization is still unknown.


In general for the coenzyme B$_{12}$-dependent rearrangements a radical mechanism is proposed, as was recently summarized by Rétey$^{14}$, based upon data arising from isotope labeling$^{15,16}$ electron paramagnetic resonance$^{17-19}$ and UV/VIS spectroscopic measurements$^{20}$. As Rétey describes in this

![Figure III.4](image)

*Figure III.4. Retention of configuration in the radical mechanism for the methylmalonyl-Co A rearrangement as proposed by Rétey.*

62
article\textsuperscript{14}, this radical mechanism is put forward independently of the stereochemical results published, while in particular the steric course of a reaction is extremely useful to draw conclusions as to the mechanism. In order to integrate the stereochemical results known and the radical mechanism, he assumes\textsuperscript{14,21} a very intimate and unambiguous interaction between enzyme and substrate, that prevents rotation around the C\textsubscript{1}-C\textsubscript{3} bond (see Figure III.4.).

While the Retey model emphasizes the role of the enzyme, it does not take into account the intrinsic properties of the substrate, i.e. an enzyme can only lead a substrate through a reaction-path that is pre-set in (allowed by) the substrate. Moreover, no explanation is given of the modifications of the enzyme needed to achieve inversion in the methyl aspartate isomerization\textsuperscript{22}.

From enzymatic data it has been shown by Pratt\textsuperscript{23}, that the three groups of rearrangements demonstrate some remarkably distinct features. The enzymes of the carbon-skeleton rearrangements require no other cofactors\textsuperscript{23}, while those of amine migrations all apparently require pyridoxal phosphate and sometimes other cofactors such as K\textsuperscript{+}, Mg\textsuperscript{+} and ATP. The enzymes of the hydroxyl migrations all require simple ions such as K\textsuperscript{+}. Although the role of some of those factors, e.g. pyridoxal phosphate, is uncertain, the question is raised whether there is a common denominator to the mechanism of reaction of the different groups of substrates.

Another indication that the three groups of coenzyme B\textsubscript{12}-dependent reactions differ originates from ESR spectra\textsuperscript{24}. The enzymes that catalyze the isomerization of diols, glycerol ethanolamine and the reduction of ribonucleotides give very unusual and characteristic ESR spectra in the presence of substrates (i.e. when frozen during catalysis) or substrate analogues and even, in the case of ethanolamine ammonia lyase, in the absence of any substrate or substrate analogue\textsuperscript{25,26}.

In all cases, the ESR spectrum consists of two components, namely a broad resonance at \( g \approx 2.3 \) due to the Co(II) ion and a narrow doublet centered about \( g = 2 \) due to an organic
radical, the splitting being explained by interaction with the Co(II) ion. The fact that these paramagnetic species may account for up to 65% of the coenzyme present depending on the enzyme and the substrate and, where detectable, are formed at a rate comparable to, or greater than, the turnover number of the enzyme strongly suggests that they represent intermediates in the catalytic cycle\textsuperscript{25}. No such signal could, however, be observed with methylmalonyl-Co A mutase\textsuperscript{27}.

Though a radical mechanism might be appropriate for hydroxyl and amine migrations, the probability of such a mechanism with respect to the carbon-skeleton rearrangements becomes less.

The only article published to date indicating a free radical rearrangement involving the [1,2]-migration of a thioester group as a model for the methylmalonyl Co A mutase reaction, is recently published by Halpern et al\textsuperscript{28}. This article, however, is not very convincing. The kinetic measurements are presented in such a way that radical and anionic processes are not directly comparable. As far as one can see from the data presented, the uncatalyzed rearrangement rate of the radical is 235 sec\textsuperscript{-1} at 60.5°C and 2.5 sec\textsuperscript{-1} at 30°C, while \( k_{\text{cat}} \) for the enzymatic methylmalonyl-Co A reaction has been estimated to be \( 10^2 \) sec\textsuperscript{-1}. Rearrangement of the anion gave after 2 min. more thioester rearranged product (42% versus 1-9% in the radical rearrangement) at a lower temperature (-78°C versus 30°C) which comes closer to the enzymatic process than the radical rearrangement. Moreover, no reason is given why Halpern considers the formation of such a substrate carbanion to be highly unfavorable and much less likely than the alternative free radical rearrangement, although contributions from pathways involving carbanion intermediates cannot be definitively excluded!

Not only there is lot of literature suggesting the three groups of carbon-skeleton rearrangements have different mechanisms of action, there is also accumulating evidence\textsuperscript{29-38} that in methylmalonyl-Co A mutase reactions the Co-C bond assists in the formation of a substrate carban-
ion in the rearrangement step. In the next Chapter we will try to make plausible, why this could be the mechanism of action for all the carbon-skeleton rearrangements.

III.3 The stereochemistry of the carbon-skeleton rearrangements as 'test' for the carbanionic mechanism. Scope of the second part of this thesis.

The few attempts made to date to include the stereochemical results known for the carbon-skeleton rearrangements in the back-side ethylene glycol

Front-side

Figure III.5. The stereochemistry of the methylmalonyl-Co A and methyl-aspartate rearrangement, according to the mechanism proposed by Corey et al.
mechanisms proposed, were incomplete (Rétey14, see Chapter II.2.) or simply incorrect (Corey et al29). The keystep Corey proposes for the inversion of configuration in the conversion of ethylene glycol to acetaldehyde29 is the migration of a hydroxylgroup (with electron pair) to an electrophilic adjacent carbon and simultaneous bonding of cobalt (through its vacant co-ordination side) to the methylene carbon in a back-side displacement of OH (see Figure III.5.). This back-side displacement will lead to inversion of configuration. If we suppose that this mechanism is also applicable to the carbon-skeleton rearrangements, then the -COSCo A migration in the methylmalonyl-Co A isomerization to a nucleophilic adjacent carbon and simultaneous bonding of cobalt to the methylene carbon in a front-side displacement of the -COSCo A group explains the retention of configuration found for this rearrangement. However, the -CHNH2COOH group in the methyl-aspartate isomerization migrates to the same nucleophilic carbon as found for the methylmalonyl Co A rearrangement, which is in contradiction to the inversion of configuration known for the methylaspartate isomerization.

As indicated in Chapter II.2. model studies suggest29-38 that in methylmalonyl-Co A mutase reactions the Co-C bond assists in the formation of a substrate carbanion in the rearrangement step. If this model description (carbanion-formation) could predict the stereochemistry of the carbon-skeleton rearrangements in general, this could be considered as an extra indication, in addition to the literature known to date29-38, towards a rearrangement of a anionic intermediate. So the scope of this part of the thesis is to find a correlation between the evolution of the coefficients of the atomic orbitals in the Highest Occupied Molecular Orbital (HOMO) of anionic intermediates, and the stereochemistry known for these reactions.
III.4 The nature of the hydrogen transferred temporarily to coenzyme $B_{12}$ during the carbon-skeleton rearrangements.

The way in which the anions can be generated is subject to a lot of speculation. Three possible ways are summarized below. All three should obey the observation of Miller et al., showing the hydrogen which migrates during the isomerization of methylmalonyl-Co A to succinyl-Co A becomes one of three equivalent hydrogens on $C_5'$ of coenzyme $B_{12}$, before a hydrogen is returned to the substrate. The first two, suggested by those who hold to initial radical generation for all the coenzyme $B_{12}$-dependent rearrangements consist of radical generation in the substrate, followed by either electron transfer from cobalt to the substrate:

$$R\cdot + Co(II)\rightarrow Co(III)^+ + R^-,$$

or by charge transfer from protein basic and acidic sites to the substrate radical, a suggestion put forward by Finke. A third possibility is proton loss from the substrate to $C_5'$ of the coenzyme (see Figure III.6.), whereby one of the three hydrogens of the substrate methyl group originated becomes covalently bonded to cobalt via an agostic $M(H)C$ interaction, as proposed by Brookhart et al. They state that carbon-hydrogen bonds, especially those of saturated (sp$^3$) carbon centres, are normally regarded as being chemically inert. Generally, the C-H group is not thought of as a potential ligand which can have a structural role or play an energetically
significant part in ground states or in reaction intermediates. They review recent observations which show that there are in fact many circumstances in which a carbon-hydrogen group will interact with a transition metal centre with formation of a two-electron three-centre bond and that the effect of the interaction is such as to have a marked effect on the molecular and electronic structure and hence reactivity of the molecule. Once the anionic intermediate is formed, substrate-enzyme interaction can accommodate for the charge build-up in the substrate at the various stages of the rearrangements.
References and notes.

41. Personal communication of R.G. Finke.
IV. Quantum chemical calculations.

IV.1 Introduction.

In order to test the model description, suggesting the formation of a substrate carbanion in the rearrangement step (see Chapter III.), for the stereochemistry of the carbon-skeleton rearrangements in general, we selected the anionic cyclopropane intermediates as given in Figure IV.1. for the quantum chemical calculations.

\[
\begin{align*}
\text{with } R &= \begin{cases} 
\text{methyl-} & \text{SCoA} \\
\text{malonyl-Co A} & \text{methyl-aspartate} \\
\text{isomerization} & \text{methyl-itaconate} \\
\text{isomerization} & \text{isomerization}
\end{cases}
\end{align*}
\]

Figure IV.1. The anionic cyclopropane intermediates which account for the stereochemistry of the carbon-skeleton rearrangements.

For sake of simplicity the calculations were confined to the substrate system without introducing enzyme or coenzyme specific sites. The key intermediate as illustrated in Figure IV.1. is formed by proton abstraction from the methyl group.
(C₃) followed by an approach of group R and the now negatively charged methylene group. The intermediate ring closure during the isomerization of methylmalonyl-Co A and methylitaconate is facilitated by polarization of the C=O and C=C bond in group R respectively. In the isomerization of methylaspartate a keto-enol tautomerization can provide an analogous structure, able to accommodate negative charge (see Figure IV.2.)

![Figure IV.2. The keto-enol tautomerisation in methylaspartate resulting in a structure which accommodates the negative charge after ring closure.](image)

Instead of one enzyme essential for the isomerization of methylmalonyl-Co A and methylitaconate, the enzyme complex for the isomerization of methylasparate consists of two proteins. The fully optimized MNDO structure of the enol form is only 2 kcal/mole higher in energy than the keto form, an energy difference which is smaller than the unpredictable error of the MNDO method¹. The final rearrangement product is formed by proton addition at the acid substituted C₁ and rupture of the C₁-R bond.

IV.2. The choice of the calculational method.

As indicated by McIver and Komornicki², a detailed understanding of the dynamics and stereochemistry of organic
reactions requires, above all, a knowledge of the many dimensional potential energy surface. The very dimensionality of this surface, however, precludes its evaluation for all but the simplest systems. To reduce this problem to one of a tractable size, two general approaches have been employed. The first type of approach seeks to reduce the dimensionality of the surface by eliminating certain degrees of freedom, for example the length of a carbon-hydrogen bond. A related technique involves choosing one or two degrees of freedom as independent variables of the potential energy and to allow the system to relax by optimizing the remaining degrees of freedom for each value of the independent variables.

The second type of general approach involves consideration of all the degrees of freedom of a system, but seeks only to locate certain chemically interesting points on the potential energy surface. For a one-step reaction, these points would be the local minima corresponding to the equilibrium geometries of reactants and products and a col or saddle point which separates the local minima.

For a number of reasons this last method, which is preferred by the author of the cited article, cannot be followed in this case. The main reason is, that our approach to the problem is a dynamic one. Just localizing stationary points on the curve would not elucidate effects such as fast electron flow and orbital inversion (vide infra). In the 'product' minimum these effects will be damped out, while comparison of the points on the reaction path somewhere in between the transition state (whose exact position is not important here) and the 'product' minimum (called 'End' further on in the article) will give the desired information. An extra handicap is the definition of a stationary point at the 'Start' (vide infra) of the ring closure. 'Start' is defined as the linear molecule with tetrahedral carbon angles just after proton abstraction. This is not a stationary point, but a rather unstable intermediate. Reasons why is chosen for the first type of approach indicated above.
IV.3. Results

The formation of the cyclopropane intermediates of Figure IV.3. has been studied with MNDO calculations.

Figure IV.3. The three intermediates of which the ring closure has been studied.

Of course MNDO results cannot give the final proof of the assumed reaction mechanism, but a detailed understanding of the dynamics and stereochemistry of organic reactions requires, above all, a knowledge of the potential energy surface. The energy profile of all three ring closure reactions is calculated by optimization of all distances, angles and torsion angles to minimal heat of formation at a number of fixed values of \( r_1 \) and \( r_2 \) (see Figure IV.3.), ranging between 1.35 Å and 1.80 Å for \( r_1 \) and between 1.40 Å and 2.50 Å for \( r_2 \). In this way the angle \( \mathrm{C}_3-\mathrm{C}_1-\mathrm{C}_2 \) changes from tetrahedral (in the linear molecule direct after proton abstraction) to triangular (at the end of the ring closure). As initial values for distances and bond angles, those optimized by Dewar et al. for the MNDO program are used. The reaction path is drawn along the line of minimal energy. The results of these calculations are given in the Figures IV.4., IV.5. and IV.6. Following the reaction path, the heat of formation as a function of the reaction co-ordinate \( r_2 \) is given for all three ring closure reactions in Figure IV.8. The graphs are closely related, except that the transition state of the
Figure IV.4. Heat of formation in kcal/mole as a function of $r_1$ and $r_2$ for intermediates during ring closure for the methylmalonyl-Co A isomerization.
Figure IV.5. Heat of formation in kcal/mole as a function of $r_1$ and $r_2$ for intermediates during ring closure for the methylaspartate isomerization.
Figure IV.6. Heat of formation in kcal/mole as a function of $r_1$ and $r_2$ for intermediates during ring closure for the methylitaconate isomerization.
Figure IV.7. Heat of formation in kcal/mole as a function of the reaction co-ordinate $r_2$ for the three carbon-skeleton rearrangements.
methylitaconate ring closure is situated later on the reaction co-ordinate. A minimum in energy is reached for values of \( r_2 \) between 1.60 and 1.50 Å. The calculations are not extended beyond this value because in our model the proton addition to \( C_1 \) takes place before this minimum in energy is reached. The charge density accumulated on the various groups of the intermediate structures varies with the reaction co-ordinate \( r_2 \) in a quite similar way for the three rearrangements, with the exception of the charge density on the group which stabilizes the negative charge by polarization of a double bond, i.e. \( C=O \), \( C=C(OH)_2 \) and \( C=CH_2 \) (\( C=X \) in Figure IV.8.). In the isomerizations of methylaspartate and methylitaconate, the charge accommodated by this group in the beginning of the reaction co-ordinate is high in comparison with the isomerization of methylmalonyl-Co A, as can be seen in Figure IV.8.

* Methylmalonyl-Co A
* Methylaspartate
* Methyllitaconate

Figure IV.8. Charge on group \( =X \) as a function of \( r_2 \) for the carbon-skeleton rearrangements.
The charge distribution over the intermediate structures at different stages of the ring closure is given in Figure IV.9. The definition of the intermediates Start, TS (transition state) and End is given in Figure IV.7. The evolution of the coefficients of the atomic orbitals in the HOMO of the cyclopropane intermediates along the reaction co-ordinate is followed. The two atomic orbitals with the highest coefficient in the HOMO are given in Figure IV.10 as a function of $r_1$ and $r_2$.

In all three reactions the overall picture is the same. In the beginning of the ring closure the contribution of the atomic orbitals on $C_3$, the carbon from which the proton is abstracted, is dominant. After the transition state the atomic orbital on the formerly double bonded oxygen

![Figure IV.10. The two atomic orbitals with the largest coefficient in the HOMO for all three carbon-skeleton rearrangements.](image)
respectively carbon, in the direction of C₃ becomes important. Near the end of the ring formation the orbital on C₁ in the direction of C₂ will have a large coefficient in the HOMO, as can be seen in Figure IV.10. A very important difference between the three reactions becomes clear, if also atomic orbitals with smaller coefficients in the HOMO are taken into account. If the intermediate is situated in the y-z plane, with the C₁-C₂ bond on the y-axis, the contribution of the atomic orbitals of the three carbons constituting the ring is given in Table IV.1. for r₁=1.7 Å and r₂=1.6 Å.

<table>
<thead>
<tr>
<th></th>
<th>methyl-</th>
<th>methyl-</th>
<th>methyl-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>malonyl-Co A</td>
<td>aspartate</td>
<td>itaconate</td>
</tr>
<tr>
<td>C₁</td>
<td>s</td>
<td>-0.15</td>
<td>-0.12</td>
</tr>
<tr>
<td></td>
<td>x</td>
<td>+0.08</td>
<td>+0.03</td>
</tr>
<tr>
<td></td>
<td>y</td>
<td>+0.50</td>
<td>+0.31</td>
</tr>
<tr>
<td></td>
<td>z</td>
<td>-0.30</td>
<td>-0.20</td>
</tr>
<tr>
<td>C₂</td>
<td>s</td>
<td>+0.03</td>
<td>+0.04</td>
</tr>
<tr>
<td></td>
<td>x</td>
<td>+0.01</td>
<td>+0.03</td>
</tr>
<tr>
<td></td>
<td>y</td>
<td>-0.09</td>
<td>+0.07</td>
</tr>
<tr>
<td></td>
<td>z</td>
<td>-0.21</td>
<td>+0.03</td>
</tr>
<tr>
<td>C₃</td>
<td>s</td>
<td>+0.11</td>
<td>+0.06</td>
</tr>
<tr>
<td></td>
<td>x</td>
<td>+0.00</td>
<td>+0.00</td>
</tr>
<tr>
<td></td>
<td>y</td>
<td>-0.31</td>
<td>-0.18</td>
</tr>
<tr>
<td></td>
<td>z</td>
<td>+0.22</td>
<td>+0.08</td>
</tr>
</tbody>
</table>

Table IV.1. The coefficients of the atomic orbitals of the ring carbons at the moment of proton addition to the cyclopropane intermediates.
The coefficients of the atomic orbitals of C₁ and C₂ are of opposite sign in the y-direction and of equal sign in the z-direction in the methylmalonyl-Co A intermediate. Both indicate an overlap between the atomic orbitals on C₁ and C₂, as can be seen in Figure IV.11., i.e. the HOMO has a bonding character between C₁ and C₂, the electron density between C₁ and C₂ is high. In the methylaspartate and methylitaconate intermediates, the contributions of the atomic orbitals of C₁ and C₂ to the HOMO are of equal sign in the y-direction and of opposite sign in the z-direction, i.e. the HOMO has an anti-bonding character between C₁ and C₂. There is a node in electron density between C₁ and C₂. The two orbitals just below the HOMO in energy do not play an important role in the picture between C₁ and C₂.

methylmalonyl-Co A

methylaspartate

methylitaconate

Figure IV.11. Bonding and anti-bonding character of the C₁-C₂ bond in the HOMO.

IV.4. Discussion.

Further examination of the pattern given by the way charge delocalizes in the model anionic intermediate (intermediate Start in Figure IV.9.), shows that the negative charge is best stabilized on the formerly double bonded oxygen in
methylmalonyl-Co A, in comparison with the way the \(-\text{C(OH)}_2\) group accommodates the negative charge in methylaspartate. The methylene group of methylitaconate behaves intermediate in accommodation of the negative charge. The relative small initial accommodation of negative charge by the diol group in methylaspartate, can be considered as a prerequisite for a larger charge migration during ring closure. Such a large charge migration from C_3 over C_2 to C_1 might force the electron density to pass momentarily beyond C_1. Subsequent orbital inversion at C_2 will prevent the electron density to flow back via the C_1-C_2 bond and to delocalize over the cyclopropane ring. Proton addition at that moment on the reaction co-ordinate will lead to inversion of configuration on C_1, i.e. the proton comes in at the opposite side of the leaving glycyl group. The relative small charge migration during ring closure of methylmalonyl-Co A will not be strong enough to force the electron density to pass C_1, resulting in retention of configuration at C_1. This rather new concept of orbital inversion to prevent electron back-donation to C_2 is strongly supported by the picture originating from the development of the coefficients of the atomic orbitals in the HOMO. Figure IV.10. shows charge migration via C_2 and not directly from C_3 to C_1. This direction of migration is necessary for the electron density to pass C_1 in line of the C_1-C_2 bond, which will lead to inversion of configuration on C_1. Figure IV.11. indicates an anti-bonding character for the C_1-C_2 bond of the methylaspartate intermediate, which prevents the electron density to flow back to C_2 and the proton to add to the C_1-C_2 bond. The bonding character of the C_1-C_2 bond in the methylmalonyl-Co A intermediate will cause proton addition with retention of configuration at C_1, due to the high electron density between C_1 and C_2. The anti-bonding character of the C_1-C_2 bond in the HOMO of the methylitaconate intermediate suggests inversion of configuration in this rearrangement. Finally it may be of interest to note that in the case of methylmalonyl-Co A mutase using ethylmalonyl-Co A as substrate instead of methylmalonyl-Co A only
partial inversion is observed. Besides the role of the enzyme in the enzyme-substrate binding (ethylmalonyl reacts at only one thousandth the rate of the natural substrate), the loss of stereospecificity in the case of ethylmalonyl-Co A may be also electronic in nature.

IV.5. Conclusion.

Energy profiles of the carbon-skeleton rearrangements with cationic or radical intermediates in the rearrangement step are required to discuss a possible preference for the anionic pathway. However, the fact that an anionic intermediate can explain the known stereochemistry, can be considered as an extra indicator in addition to the chemical evidence suggesting a carbanion in the rearrangement step of the methylmalonyl-Co A isomerization.
References and notes

Summary.

The first part of this thesis describes investigations concerning the role of penta co-ordinated phospholipid intermediates in the activation of membrane embedded proteins, viz. ion channels. On the basis of extensive literature search a model is drawn in which a conformational change in the phospholipid headgroup from a four co-ordinated tetragonal (P(IV)) to a five co-ordinated trigonal bipyramidal (P(V) TBP) structure results in a change of conformation of the lipid hydrocarbon region. The conformational change in the phospholipid headgroup is induced via external factors as cation concentration, potential field and binding of e.g. a water molecule. Enhanced electronegativity on the axial phosphate oxygen of the five co-ordinated intermediate, leads to repulsion of the sn-2 oxygen bound via an O-C-C-O sequence to the phosphate group. The resulting adaptation in the hydrocarbon chain region will lead to cluster formation in the sense of a region of different density compared to the surrounding matrix. Cluster formation around a membrane protein (viz. ion channel) will activate that ion channel to open. To test several aspects of this model description, phospholipid vesicles are synthetized of varying lipid composition. Over their wall an ion gradient is created, whose equilibration is followed with $^{23}\text{Na}$ NMR after addition of Gramicidin A. Leaving all the experimental conditions as constant as possible, except the lipid composition of the vesicle wall, passive ion transport was measured of different rates. The relative rate of ion transport as predicted on the basis of the described model, was established by the measurements.
The second part of this thesis uses the stereochemistry of the carbon-skeleton rearrangements dependent on coenzyme B₁₂ as a 'test' for the mechanism of action postulated for these isomerizations. An anionic mechanism is given, based on extensive literature search, suggesting an anionic mechanism for the methylmalonyl-Co A rearrangement. With the help of MNDO calculations, energy profiles are constructed for the three ring closure reactions towards anionic enzyme stabilized cyclopropane intermediates. Following the reaction path, charge distribution and migration in the substrates are monitored, as well as the evolution of the coefficients of the atomic orbitals in the highest occupied molecular orbital (HOMO) of the cyclopropane intermediates. Large charge migration as a consequence of small charge stabilization by a certain group in the anionic intermediate is found to be a prerequisite for the inversion of configuration known for the methylaspartate isomerization. Conversely small charge migration as a consequence of good charge stabilization in the methylmalonyl-Co A rearrangement will lead to retention of configuration. For the methylitaconate isomerization inversion of configuration is suggested.
Samenvatting.

Het eerste gedeelte van dit proefschrift beschrijft het onderzoek naar de rol van vijf-gecoördineerde fosfolipide intermediairen bij de activering van in het membraan opgenomen eiwitten, zoals ion-kanalen. Op basis van een uitgebreid literatuuronderzoek is een model opgesteld, waarin een conformatieverandering in de fosfolipide hoofdgroep van een vier-gecoördineerde tetragonale (P(IV)) naar een vijf-gecoördineerde trigonale bipyramidale (P(V) TBP) structuur, resulteert in een conformatieverandering in de koolwaterstofketens. De conformatieverandering in de fosfolipide hoofdgroep wordt geïnduceerd door externe factoren als kationconcentratie, potentiaalveld en de binding van bijvoorbeeld een watermolecule. Een verhoogde electronegativiteit op de axiale fosfaatzuurstof van het vijf-gecoördineerde intermediair, leidt tot afstoting van de sn-2 zuurstof, die via een O-C-C-O keten gebonden is aan de fosfaatzuurstof. De resulterende aanpassing in de koolwaterstofketens induceert cluster vorming. De term 'cluster vorming' wordt hier gebruikt voor een gebied met een dichtheid, verschillend van die van de omgeving. Cluster vorming rond een membraan eiwit (het ionkanaal) zal het ion-kanaal activeren, waardoor het iontransport start. Om verschillende aspecten van het model te testen, zijn fosfolipide 'vesicles' gesynthetiseerd met varierende lipidessoorten. Over de wand van deze vesicles is een ion-gradient gecreëerd. Het verdwijnen van deze gradient na toevoeging van Gramicidine A is gevolgd met behulp van $^{23}$Na NMR. Tijdens deze metingen zijn alle experimentele omstandigheden, behalve de lipidenummering van de vesicles, zo constant mogelijk gehouden. Zo kan iontransport gemeten worden van verschillende snelheid. De met behulp van het beschreven model voorspelbare relatieve snelheid van iontransport is bevestigd door de metingen.
Het tweede gedeelte van dit proefschrift gebruikt de stereo-chemie van de coenzyme B₁₂ afhankelijke koolstofskelet isomerisaties als een 'test' voor het werkingsmechanisme dat gepostuleerd is voor deze omleggingen. Een anionisch mechanisme wordt voorgesteld, gebaseerd op een groeiende hoeveelheid literatuur, die een anionisch mechanisme voor de methylmalonyl-Co A omlegging suggereert. Met behulp van MNDO berekeningen, zijn energieprofielen opgesteld voor de drie ringsluitingsreacties naar anionische enzyme-gestabiliseerde cyclopropaan intermediairen. Ladingsverdeling en migratie zijn gevolgd gedurende de reactie, alsook de verandering van de coëfficiënten van de atomaire orbitalen in de hoogst bezette moleculaire orbital (= HOMO) van de cyclopropaan intermediairen.

Sterke ladingsmigratie, als gevolg van een geringe ladingsstabilisatie door een bepaalde groep in het anionisch intermediair, blijkt een voorwaarde te zijn voor de inversie van configuratie, gevonden voor de methylaspartaat isomerisatie. In tegenstelling hiermee leidt een geringe ladingsmigratie, als gevolg van een goede ladingsstabilisatie in de methylmalonyl-Co A omlegging, tot retentie van configuratie. Voor de methylitaconaat isomerisatie wordt op grond van de berekeningen een inversie van configuratie verondersteld.
Curriculum vitae.


Van 1 december 1980 tot 1 mei 1985 was zij werkzaam bij de vakgroep Organische Chemie van de Technische Hogeschool Eindhoven als wetenschappelijk assistente. Tijdens deze periode werd het onderzoek, beschreven in dit proefschrift, uitgevoerd onder leiding van Prof. Dr. H.M. Buck.
Dankwoord.

Gedurende het onderzoek, dat geleid heeft tot dit proefschrift, heb ik van velen steun ondervonden. Alhoewel ik niet allen bij naam kan noemen, wil ik toch enkele van hen zeer specifiek bedanken.

Als eerste wil ik bedanken het technisch en administratief personeel, zowel binnen als buiten de vakgroep. Zij hebben mij geholpen de in tijden van bezuiniging verouderde apparatuur op te knappen.

Ten tweede wil ik bedanken degenen die mij moreel ondersteund hebben, waarbij ik mijn echtgenoot, familie, vrienden en de koffie-club uit de rookvrije kantine graag expliciet wil noemen.

Bij het tot stand komen van de $^{23}$Na NMR heb ik steun ondervonden van Jan de Haan en Leo van de Ven.

Bij het rekenwerk heb ik veel steun ondervonden van Maarten Donkersloot. Hanneke Becht wil ik bedanken voor het werk dat zij tijdens haar afstuderen op het gebied van Vitamine $B_{12}$ heeft verricht.

Tenslotte wil ik Ria Hoozemans hartelijk bedanken voor het typewerk, dat leidde tot de uiteindelijke vormgeving van dit proefschrift. De mogelijkheid die zij creëerde, dit proefschrift met behulp van de tekstverwerker tot stand te laten komen, gaf mij de gelegenheid deze laatste fase van de promotie overeenkomstig de eisen van deze tijd te volbrengen.

De bijzonder fraaie tekeningen in dit proefschrift zijn gemaakt door Henk Eding en Cas Bijdevier.

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Stellingen.

1. De bewering van Roberts en medewerkers dat de $3p_x$ en de $3p_y$ orbital deel uitmaken van de enkel bezette MO in TBP fosforanylradicalen, moet op grond van symmetrie overwegingen verworpen worden.

   J.W. Cooper, M.J. Parrott en B.P. Roberts,

2. Modelverbindingen voor koolstofskelet isomerisaties onder invloed van Vitamine B$_{12}$, waarin het substraat gebonden is aan het centrale cobaltatoom, kunnen niet worden gebruikt om conclusies te trekken met betrekking tot waterstof abstractie van niet geactiveerde koolstofatomen.


3. Het is de vraag of de ontwikkeling van nat naar droog ontwikkelbare lakken in de lithografie ten behoeve van de I.C. technologie, analoog aan de ontwikkeling van nat naar plasma etsen, zal leiden tot een betere patroondefinitie.

4. Het inschatten van de mogelijkheid korter te gaan werken en een deel van de eigen functie over te dragen aan anderen, is bij leiding geven de functionarissen mede afhankelijk van het vermogen de eigen complexe functie te analyseren en van het vermogen tot delegeren.

   Mr. H. Luik, Intermediair 35, augustus 1984, 1-5.

5. De conclusie van Bock en medewerkers, dat bij de isomerisatie van D-fructose naar D-glucose onder invloed van het enzym glucose-isomerase,
het α-anomere bij het begin van de omzetting met een hogere preferentie gevormd wordt dan uit de evenwichtstoestand blijkt, is een overschatting van de mogelijkheden van de NMR techniek.

K. Bock, M. Meldal, B. Meyer en L. Wiebe,

6. De constatering dat sommige proteïnen goed functioneren in een milieu bestaande uit enkel detergentia, behoeft niet in strijd te zijn met de bewering dat membraanproteïnen een specifieke lipide-omgeving nodig hebben om geactiveerd te worden.

P.R. Cullis en B. de Kruijff, Biochim. Biophys..Acta, 1979, 559, 399-420.

7. De 'closed-shell' 1A_1 overgangstoestand met C_{2v} symmetrie van de thermische [1,5] - H shift in pentadiën, zoals berekend door Hess en Schaad, is een aangeslagen toestand en niet een grondtoestand.


8. Het wekt op zijn minst enige verwondering, dat het vrijkomen van formaldehydgas uit spaanplaat bekend is en tot klachten leidt bij een aanzienlijk gedeelte van de Nederlandse bevolking, terwijl de aanwezigheid van ditzelfde gas in sigaretterook systematisch genegeerd wordt.

9. Bij de productie van (Al, Ga) As - halfgeleiderlasers, geschikt voor toepassing in 'compact-disk' spelers, met behulp van organometaalgasfase epitaxie, verdient het aanbeveling meer aandacht te besteden aan de epitaxiale groei van (Al, Ga) As - lagen waarbij de groeisnelheid kinetisch bepaald is.
10. Bij de bestudering van conformatieveranderingen in DNA structuren, die optreden ten gevolge van complexering met metaalionen, wordt een onjuist beeld van de fosfaat-metaal complexatie verkregen, indien gebruik wordt gemaakt van 5'-nucleotiden die enkelvoudig zijn veresterd op de fosfaatgroep.


11. Bedrijven en overheidsinstellingen zullen via de wet gedwongen moeten worden om een bepaald percentage vrouwen in dienst te nemen.

Mr. F.H.A.M. Kruse, directeur-generaal Arbeidsvoorzieningen, Ministerie van Sociale Zaken, januari 1985, op het congres 'Wilma wil werk'.

Ir. I.I. Merkelbach,
10 mei 1985.