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BIOPHYSICAL CHEMISTRY AT HIGH PRESSURE

By K. Heremans

This paper is concerned with new developments in instrumentation together with a discussion on pressure effects on hydrogen bonding, electrostatic and hydrophobic interactions in water. The main part is on the pressure effects on transitions in proteins and phospholipids. A final section is on the interaction of lipids with proteins in biomembranes.

1. Introduction

The application of physical chemical techniques to the study of biological molecules has resulted in new fields of research which have been called: biophysics, biophysical chemistry, physical biochemistry, *etc.* An outsider might get confused, but it is clear that the laws of physics and chemistry are also valid in the biological world.

A unique feature of the chemistry in the biological world is the role played by macromolecules such as proteins, nucleic acids, polysaccharides and supramolecular structures such as membranes, ribosomes, chromatin, *etc.* All these processes go on in water, a rather unique solvent due to its intermolecular hydrogen bonding.

The primary role played by noncovalent interactions in these systems makes them attractive for pressure studies. The initial experiments by Bridgmann¹⁾ in 1914 gave the impression that the only effect of pressure on proteins would be to destroy their structure by a process called denaturation. Suzuki, Miyosawa and Suzuki²⁾ however were the first to show, with optical techniques, that a careful choice of the experimental conditions brings about reversible changes in proteins. In contrast nucleic acids were found to be very pressure resistant. The results of these researches are reviewed in a paper which is probably one of the most cited articles in the field of high pressure effects on biopolymers.³⁾ These authors have also used a number of model systems in order to get information on the volume changes for molecular interactions which play a role in the stability of proteins and nucleic acids.

Much of the work in this field originated in an article written by Kauzman⁴⁾ in 1959 on protein denaturation.

In this work we review shortly developments in high pressure instrumentation. We discuss the volume changes for noncovalent interactions (model systems). The main part is on pressure effects on proteins, with a section on hemoproteins: the interaction of proteins with small

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molecules as well as with proteins themselves to form supramolecular structures. We finally discuss phospholipids and their interaction with proteins in membranes. Some of the material has been recently discussed by the author.^{5,6)} Pressure effects on enzyme reactions are not included.

2. Developments in Instrumentation

In this section we only concentrate on new developments in techniques applied to the study of biological systems. Hawley⁷⁾ has recently reviewed the techniques to measure volume changes, gel electrophoresis and optical measurements under pressure. Since then a high pressure cell has been described⁸⁾ for circular dichroism studies in the low pressure range. Optical rotation measurements have been done in the low⁹⁾ and the high¹⁰⁾ pressure range. Changes in fluorescence polarization have been measured by Chrysomalis, Drickamer and Weber.¹¹⁾ Dilatometry¹²⁾ has been used to study the phase transitions in lipids. A versatile high pressure chamber has been described for electrophysiological experiments up to 300 atm.¹³⁾

The field with respect to fast reaction techniques has been reviewed elsewhere.¹⁴⁾ Since then several new techniques have been adapted for work under pressure. NMR studies on biopolymers have been done by Williams, Fyfe, Bruck and Van Veen¹⁵⁾ and by Gaarz and Lüdemann¹⁶⁾ with glass capillary tubing originally introduced by Yamada.¹⁷⁾ Merbach and Vanni¹⁸⁾ have used a different approach which is very promising for high resolution work. 360 MHz H NMR spectra have recently been obtained with glass capillary tubing with long scanning times.¹⁹⁾

le Noble and Staub²⁰⁾ have used glass capillary tubing for EPR work which opens up the field for spin label studies in biomembranes.

Progress has also been made by the introduction of stopped-flow instruments for work up to 1 kbar²¹⁾ and 3 kbar.²²⁾ A pressure jump apparatus has been developed for work up to 1.5 kbar.²³⁾ A nanosecond temperature-jump apparatus has been described by Liphard.²⁴⁾ All these methods considerably extend our time scale for the study of biochemical reactions under pressure. Recently, dynamic light scattering experiments have been made up to 500 bar.²⁵⁾ Halvorson²⁶⁾ has described the application of pressure perturbation in the time and frequency domain for the study of the self assembly of proteins.

Finally two recent papers remind us that not only high pressure techniques give us information on the volumetric behaviour of biomolecules: From the concentration dependence of the specific volume, information can be obtained on the volume change of protein-protein interactions.²⁷⁾ From ultrasonic measurements,²⁸⁾ one obtains the adiabatic compressibility of proteins together with its time and frequency dependence.

3. Intermolecular Interactions

Kauzmann²⁹ has discussed the importance of hydrogen bonding, electrostatic and hydrophobic interactions, responsible for protein stability. These interactions play a role in a wide variety of biochemical reactions going on in water as a solvent. They are affected to a different extent by pressure and we will review these effects briefly with reference to Table 1.

Volume changes for hydrogen bonding have been estimated at -5 ml.³¹ The recent work by Josefiak²⁹ has shown that -2 ml is a better value. These values are obtained in nonpolar solvents. In aqueous solutions these values must be close to zero since a hydrogen bonded solvent molecule is exchanged for another molecule.

Electrostatic interactions are much more pressure dependent owing to the solvent electrostriction around free ions. The field has been reviewed by Hamann.³⁰ A practical consequence of pressure effects on ionic equilibria is that one has to be careful when choosing buffer systems. Tris buffer is nearly ideal in this respect while phosphate buffers are highly pressure dependent.³¹ One should also realize that temperature affects these buffers just in the opposite way!

Numerical values for the volume changes of hydrophobic interactions have been discussed

Table 1. Volume changes for intermolecular interactions in water

Interaction	ΔV (ml/mol)	Ref.
Hydrogen bonding		
Self-association of ϵ -caprolactam	-0.65	29
Self-association of phenol	-2.3	29
Electrostatic		
$\text{HPO}_4^{2-} + \text{H}^+ = \text{H}_2\text{PO}_4^-$	$+24$	31
$\text{Tris} + \text{H}^+ = \text{TrisH}^+$	$+1$	31
Formation of salt bridge in Chymotrypsin	$+30$	39
Hydrophobic		
—Aliphatic chains		
Dimerization of carboxylic acids Methyl	$+1$	40
Ethyl	$+5$	40
Propyl	$+8$	40
Coil-to-helix transition of Poly-L-glutamic acid	$+1/\text{residue}$	41
Partial molar volumes of alcohol-water solutions	$+1/\text{CH}_2$	42
—Aromatic rings (stacking processes)		
FMN+DMP	-4.8	43
Flavinyltryptophan peptides	-1.8 ($n=3$)	34
	-4.8 ($n=5$)	34
DNA bases	-4 to -7	16, 44
Dyes Proflavin	-6	45
Biebrich Scarlet	-12	46

in a number of papers.^{3,5,16,32,33,34)} A simplified picture is that aliphatic hydrocarbons show positive volume changes on association while aromatic hydrocarbons show negative volume changes.

Any quantitative model to explain the sign of the volume changes in these interactions has not been given. A simple qualitative model would be that where spheres (water) are packed around flat (aromatic) or rough (aliphatic) molecules. This crude model would at least give the right sign for the observed volume changes. Whatever the explanation may be, it seems that these effects can explain the difference between the behaviour of proteins and nucleic acids under pressure.

The role of water has been especially invoked to explain a number of observations.³⁵⁾ A recent review by Kauzmann³⁶⁾ on the structure of water warns us against the use of the structure of this compound to explain biological phenomena. A number of factors play a role in hydrophobic interactions and water is certainly one of them. But it seems at present more appropriate to concentrate on other factors in the discussion of volume changes.³⁷⁾ The role that mechanical constraints on the molecules play due to the fact that no bending of covalent bonds occurs at the pressures used in biochemical research, has been well demonstrated in the experiments with the flavinyltrypan peptides.³⁴⁾ A critical discussion of hydrophobic interactions has been given by Klapper.³⁸⁾

4. Proteins

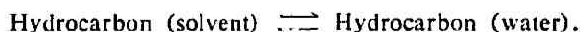
In this section we discuss conformational transitions in proteins, pressure effects on hemoproteins including spin equilibria, the interaction of small molecules with proteins and the association of proteins to form supramolecular structures.

4-1. *Conformational transitions in proteins*

Proteins are linear polypeptides folded up in a compact form called the native conformation. Under certain conditions of temperature, pH, pressure, *etc.*, this native conformation can unfold into a denatured conformation. The volume of a protein in solution is made up from three contributions⁴⁰⁾: 1) the constitutive volume, 2) the void volume due to imperfect packing and 3) the volume change due to the solvation of peptide and amino acid residues. In solution contributions 2) and 3) seem to cancel each other. Kauzmann⁴¹⁾ has discussed the parallelism between thermodynamic factors governing the process of reversible denaturation



and the dissolution of hydrocarbon molecules in water



If we accept the picture of a protein where all the hydrophobic side chains of the amino

acid residues are on the inside, then one expects that pressure would affect both processes in the same way. A number of studies have revealed that pressure effects on the reversible denaturation of ribonuclease,⁴⁸⁾ chymotrypsinogen⁴⁹⁾ and metmyoglobin⁵⁰⁾ are much smaller than expected on the basis of the behaviour of the model systems. Several explanations can be put forward for the observed discrepancies. They are related to the basic assumption in the above discussed protein model.

The first assumption is that the denaturation process can be described as a two state model, *i. e.* the model starts from the assumption that both the native and the denatured state are well defined structures.

Hawley and Mitckell⁵¹⁾ in their kinetic studies of chymotrypsinogen denaturation at pH 2, found evidence for a two state model. But the authors point out that this does not mean that the existence of low levels of intermediate states are excluded.

Li *et al.*,⁵²⁾ on the other hand, "disprove the two state hypothesis" in their thermodynamic study of chymotrypsinogen and lysozyme. Observing the protein fluorescence they find a first domain of the protein which denatures below 8 kbar. With ANS binding in the case of chymotrypsinogen and protein fluorescence in the case of lysozyme, they find a second independent domain between 8 and 11 kbar. Thus these studies reveal a plurality of pressure-denaturated forms in both proteins. The same authors⁵²⁾ find only one domain however up to 10 kbar for the riboflavin binding protein of egg white.

Brandts⁵³⁾ has pointed out that in theory any transition can conveniently be defined as a two state transition by any arbitrary division of the microscopic assembly into two parts. This approach has been used in the above discussed results. In recent years it has become clear that proteins are highly flexible structures. A recent study reveals that the activation volume for the rotational motion of internal aromatic rings in globular proteins can be as high as 60 ml.⁵⁴⁾ These measurements, among others, show that protein fluctuations provide some void volume around certain residues. It should also be noted that this activation volume is of the order of magnitude calculated for fluctuations in proteins⁵⁵⁾ and those experimentally obtained for reversible denaturation. Compressibilities of native proteins are discussed by Gekko and Noguchi.⁵⁶⁾

Another way to explain the discrepancies is to have a closer look to the model systems. As already indicated in the previous section, it is necessary to make a distinction between aliphatic and aromatic residues. These observations refine the initial picture where it was assumed that hydrophobic interactions are accompanied by large positive volume changes. The compensation due to the exposure of aliphatic and aromatic residues would then explain the small volume changes for protein denaturation. Hvidt⁵⁷⁾ has shown that hydrocarbons are not good model systems. Alcohols, ketones, amides and ethers are more closely related to protein components.

Li *et al.*³²⁾ have pointed out the possible significance of the methods used to study the denaturation. These are uv, visible and fluorescence spectroscopy and could thus reflect local changes on the protein. In other words, unfolding might not be as extensive as originally

supposed. This might explain also why insulin is stable up to 48 kbar.⁵⁸⁾ It would therefore be desirable to use the techniques which look to the proteins as a whole and not just to one spot on the surface or the interior of the molecule. On the other hand, the volume changes observed with optical methods agree rather well with those obtained from dilatometry on lysozyme⁵⁹⁾ and metmyoglobin⁶⁰⁾ and with optical rotation experiments for ribonuclease.⁵¹⁾

Perutz⁶¹⁾ has discussed the electrostatic effects in proteins and concluded that they may dominate many aspects of protein behaviour. This is very clear in the salt bridge of chymotrypsin³⁹⁾ which stabilizes the native structure of the enzyme.

The salt bridge of chymotrypsin is disrupted by pressure with a volume change of -30 ml. If the environment of the salt bridge is predominantly hydrophobic, then the volume change is largely due to the salt bridge as a consequence of the lower dielectric constant of the medium. The results must be taken into account when measuring steady-state activity of chymotrypsin under pressure. They also explain our previous findings⁴⁵⁾ that the binding of proflavin to chymotrypsin is pressure independent. The binding to the active molecule shows a normal, i.e. negative volume change.

Brandts⁴⁸⁾ and Hawley⁴⁹⁾ have made the interesting observation that proteins show a quite different pressure-temperature stability behaviour in contrast to nucleic acids. In detailed studies Hawley showed that the P - T coexistence lines for proteins show considerable curvature while nucleic acids⁶²⁾ show no curvature. Several proteins exhibit these phenomena of maximum stability at certain T and P . The situation becomes even complex when the PH is included in the analysis as shown by Zipp and Kauzmann.⁵⁰⁾ This phenomenon has been attributed to the exposure of hydrophobic groups to the solvent upon unfolding.

At low pressures, dT_m/dp is low but positive (see Table 2), while with increasing pressure dT/dp becomes negative. These observations will be further discussed in relation to pressure effects on transitions in lipids. It is interesting to note that certain liquid crystals also show elliptic phase boundaries between smectic and nematic phases. This was observed by Cladis *et al.*⁶³⁾ Nucleic acids and lipids do not show this phenomenon and one is therefore tempted to speculate that the presence of both aromatic and aliphatic groups in proteins and liquid crystals explain their peculiar behaviour.

Table 2. Pressure effects on transitions in proteins, nucleic acids, and phospholipids

	dT/dP deg/kbar	Ref.
Ribonuclease	2.2	48
Chymotrypsinogen	1.7	49
Metmyoglobin	6.5	50
Poly-benzyl-glutamate	5.6	9
AMA Helix-Coil	0.3-4	62
Phospholipids	21	64

4-2. *Spin equilibria and redox reactions in hemoproteins*

Hemoproteins are characterized by their absorption in the visible region of the spectrum. This is due to the presence of a hem group, i.e. a porphyrin ring filling four coordinates of iron, the fifth and the sixth places are provided by the protein, a ligand or solvent. The first systematic pressure study was done on myoglobin by Zipp and Kauzmann,⁵⁰⁾ who reported that high spin spectra are transformed in low spin spectra by high pressure. This work has been extended to other proteins.⁶⁵⁾ The pressure needed to convert high spin in low spin depends on the protein. The volume changes are of the order of 50 ml for the transition in cytochrome at acid pH. The volume change reflects changes in the protein structure, since similar volume changes are obtained in the alkaline region where the protein is essentially low spin.³⁹⁾ Much smaller volume changes are found in inorganic complexes. The primary effect of pressure on proteins with an open crevice might therefore be to close the crevice. Recently Morishima *et al.*⁶⁶⁾ have obtained 220-MHz NMR spectra of hemoproteins under pressure. The results are also interpreted as a shift in favour of low spin forms at high pressure.

The presence of porphyrin bound iron has two interesting consequences: ligand binding with possible changes in spin state and redox reactions.

Volume changes for ligand binding to methemoglobin and metmyoglobin have revealed that hydration changes and spin state changes, which are linked to structural variations in these proteins, are also pH dependent.⁶⁷⁾

Conflicting results have been published on the pressure effects on oxygen binding to hemoglobin. From independent experiments it is concluded that the R-T transition is not pressure sensitive.⁶⁸⁾ This again reflects subtle effects of the protein environment on the movements of the iron in the plane of the hem. This is also evident from NMR work. Activation volumes for oxygen and CO binding to hemoglobin and myoglobin have been reported by Hasinoff.⁶⁹⁾ No activation volumes have so far been obtained for fast spin equilibria in hemoproteins. A study has been made in inorganic complexes where the transition state seems to be close to the low spin form.⁷⁰⁾

Redox equilibria and kinetic studies have been done with high pressure temperature-jump and stopped flow on cytochrome c.^{14,71)} The reactions are known to be outer sphere but the activation volume is positive for the reduction with iron-hexacyanides and is negative for the oxidation. The reduction with ascorbic acid shows a negative activation volume. This reflects the influence of the total volume change on activation volume for the redox reaction.⁷²⁾ Similar arguments apply to the other activation parameters.⁷³⁾

4-3. *Interaction of small molecules with proteins*

On the basis of the models described one should be able to predict volume changes for the interaction of small molecules with proteins, if information is available about the nature of the interaction site. One then assumes that some type of interaction is predominant which is not necessarily the case. On the other hand one also assumes that pressure does not affect

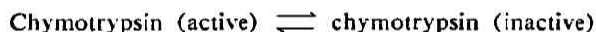
Table 3. Volume changes for the binding of ligands to proteins

Reaction	ΔV (ml/mol)	Ref.
Trypsin + Proflavin	+17	45
Chymotrypsin + Proflavin	0 (-5)	39
Chymotrypsin + Biebrich Scarlet	-5 (-10)	46
Lysozyme + ANS	-3	52
Chymotrypsinogen + ANS	-3	52
FMN + FMN binding protein	-3 (<1 kbar)	32
	+70 (>3 kbar)	32
FMN + Flavodoxin	+65	74
Concanavalin A + MUM	+25	75
Poly-cyclodextrin + ANS	+9.3	37
" " + PRODAN	+9.3	37
RNAase + S-peptide	+31	76

the protein, which leads to measured volume changes for coupled processes. These can only be separated with fast reaction techniques. A typical example is given by our study of the binding of proflavin to chymotrypsin.⁴⁵⁾ As indicated in Table 3 $\Delta V=0$ for the reaction



From other studies³⁹⁾ we know however that chymotrypsin exists in two conformations



The volume change for this equilibrium is -30 ml/mol (Table 1). Since proflavin binds only to the active conformation, a correction for the enzyme equilibrium has to be incorporated which gives:



Similar arguments can be applied to trypsin which makes the positive volume change somewhat smaller.

It can be seen from Table 3 that in almost all cases the volume change for the binding of small molecules is negative. The positive value for trypsin is due to the presence of a negative group in the binding pocket.

In a recent paper, Weber and coworkers³⁷⁾ have looked to the problem from a different point of view. They attribute the divergent behaviour of complexes under pressure to the differences in the compressibility of the protein binding sites. Pressure stabilized binding is characterized as "soft" binding sites, i.e. sites in which rotation about backbone bonds permits reduction of the site domain under pressure. "Hard" binding sites do not decrease their size when pressure is applied. In this case pressure destabilizes the binding as exemplified by their studies on polydextrin with ANS and PRODAN. In their view the consideration of relative compressibilities offers a quantitative alternative to the usual qualitative discussions in terms

of hydrophobic and other bonds. This approach is therefore very attractive. It might for instance explain the positive volume changes observed for the binding of MUM to Concanavalin A.⁷⁵⁾ The sign of this reaction is otherwise difficult to explain.

Some observed positive volume changes can however not only be explained by the presence of "hard" sites: This is the case of trypsin with proflavin⁴⁵⁾ and RNase with S-peptide interaction⁷⁶⁾ where electrostatic effects seem to be predominant.

4-4. Protein-protein association

Volume changes observed for protein-protein interactions can be treated as an extension of the interaction of small molecules with proteins. In Table 3 we have already mentioned the example of the interaction of S-peptide with RNase. Table 4 gives a more extensive list. With only two exceptions all volume changes are positive.

Temperature plays an important role as was shown by Engelborghs and coworkers⁸⁰⁾ for microtubuli and by Payens and Heremans⁸¹⁾ for beta-casein. The association of glutamate dehydrogenase has been studied in greater detail.⁸³⁻⁸⁴⁾ The table shows some typical data. It is not entirely possible to explain the volume changes and activation volumes but it is clear that the solvent plays an important role.²⁶⁾ Weber⁸⁵⁾ has suggested in relation to another system that the dead space which remains after the association forms a possible source of positive volume change together with the mechanical constraints of the interacting sites. Recently Heremans and Wauters⁸²⁾ have obtained volume changes for the interactions of chymotrypsin with trypsin inhibitor. The volume change is large and positive.

A system of great biological interest which has been studied is ribosome assembly.⁷⁸⁾ There

Table 4. Volume changes for the association of proteins

Reaction	Volume change (ml/mole)	Ref.
Myosin	+280	77
Ribosome subunits	+250 (E. Coli)	78
	+220 (A. Salina)	79
Lysozyme	—	77
tRNA synthetase + tRNA	—	77
Microtubuli elongation	+26 (35°C)	80
	+50 (15°C)	
“ nucleation	+12 (35°C)	
	+120 (15°C)	
Beta-Casein	+ <1 kbar <— (25°C)	81
	0 <2 kbar <— (4°C)	
Chymotrypsin + Trypsin inhibitor	+80	82
Glutamate Dehydrogenase	+14† (18°C)	83
	+28 (18°C)	84

† Activation volume, obtained with high pressure temperature-jump, pressure-jump and stopped flow.

is as yet no satisfactory explanation for the large and positive volume changes.⁷⁹

The idea proposed by Weber, that the free volume or dead space between associated subunits is a source of positive volume change has attractive consequences. First one expects the volume change to become smaller as a function of pressures. This has been observed for the association of Glutamate Dehydrogenase.⁸⁶ Secondly, the free volume is expected to increase with increasing temperature. If we then assume that some solvent can be trapped between the subunits, one expects that the ΔV for association will become smaller with increasing temperature. This also has been observed for Glutamate Dehydrogenase.⁸⁴ The activation volume also decreases with increasing temperature.⁸³ Similarly, increases in free volume have been observed for the diffusion of long chain organic molecules in a polymer matrix.⁸⁷

5. Phospholipids

Synthetic phospholipid vesicles have proved to be very good model systems for the study of biological membranes. De Smedt, Olbrechts and Heremans⁶⁰ have studied the effect of pressure on the transition temperatures as studied by light scattering. Some results have been summarized in Table 5. The dT/dP values are positive and large (20 deg/1000 atm) compared with the values obtained for transitions in proteins and nucleic acids as shown in Table 2. The addition of drugs, proteins and salts which in some cases shifts the transition temperature considerably, has only a small effect on dT/dP .

Goethals and Heremans⁶⁸ have recently found that the melting of charged phospholipids shows the same pressure dependence as noncharged lipids. Ceuterick and coworkers²⁵ have

Table 5. Pressure effects on phase transitions in phospholipids

Phospholipid	T_i at 1 atm	$dT_i/dP^\dagger$	ΔT_i	Ref.
Dilauroyl-lecithine	0.5	17	—	64
Dimyristoyl-lecithine (DML)	24	20.5	—	64
Dipalmitoyl-lecithine	41.5	21.8	—	64
Dilauroyl-phosphatidyl-ethanolamine	31	21.5	—	64
DML + Na tetraphenylborate	14.9	20.5	-9.1	64
DML + chlorpromazine	21	21	-3	64
DML + cholesterol	23	20	-1	64
DML + cetyltrimethylammoniumbromide	25	20	+1	64
DML + $\text{UO}_2(\text{NO}_3)_2$	25.5	20	+1.5	64
n-C ₁₈ alkanes	28	25.7	—	91
DML + 10% phosphatidylserine (PS)	27.1	22	—	92
DML + polylysine	27	22.9	—	92
DML + PS + cytochrome C	28.4	21	+1	92
DML + PS + gramicidine	24.4	21	-3.6	92

[†] K/1000 atm

found with quasielastic light scattering, that there is no change in the outer dimensions of the lipid vesicles on going through the transition either by changes in temperature or pressure.

Macdonald¹²⁾ has observed that the width as well as the volume change of the transition is pressure independent up to 300 atm. Stamatoff and coworkers⁸⁹⁾ made x-ray diffraction measurements of lipids as a function of pressure.

From the pressure dependence of the melting of branched hydrocarbons²⁰⁾ we predict that the pressure effect on branched lipids will be the same as for unbranched lipids. A similar argument applies to the melting of unsaturated lipids.

It is interesting to compare the large pressure effects on lipids with the effects observed in proteins. (See Table 2). Also dT/dP is pressure independent up to 3500 atm. This is in contrast to the observed curved diagrams for liquid crystals and proteins. Both proteins and liquid crystals contain aliphatic and aromatic groups and one is therefore tempted to transpose the model proposed by Cladis *et al.*⁶³⁾ for liquid crystals behaviour to proteins.

6. Lipid Protein Interactions

The currently held view of a biological membrane is that a lipid bilayer is the basic matrix in which proteins are either embedded (intrinsic) or onto which proteins are attached at the outside (extrinsic). Much research effort has gone to the intrinsic proteins.⁹³⁾

An attractive concept to many reseachers is that immobilized lipids form a boundary around intrinsic proteins. The physical state of these boundary lipids controls the activity of the enzyme.

Biphasic Arrhenius plots of membrane bound enzymes have been interpreted as phase changes or phase separations in the lipid surrounding the enzyme. However literature reports can also be found where biphasic Arrhenius plots are not ascribed to lipids. It is clear from the previous discussion on temperature and pressure effects on proteins and lipids, that the study of the effect of pressure on the temperature at which the break occurs in biphasic plots, is a possibility to distinguish between both hypotheses. It should however be clear that when large effects are observed this by no means excludes a change in conformation of the protein. What these experiments indicate is whether changes in activity of the enzyme are controlled by physical changes in the state of the lipids or whether they are only controlled by the protein without the involvement of lipids. Ceuterick and coworkers⁹⁴⁾ have studied the pressure effects on the biphasic Arrhenius plots of Nitrogenase. The results indicate that lipids are involved. The authors also present biochemical evidence. De Smedt and coworkers⁹⁵⁾ have studied $\text{Na}^+\text{K}^+\text{ATPase}$ of pig kidney and observed essentially the same behaviour with ATP as substrate.

Interestingly, the *p*-nitrophenylphatase activity shows no biphasic behaviour and no biphasic plots were observed for the activity as a function of pressure. This indicates that the activity of the enzyme towards this substrate is not controlled by lipids. Macdonald and Macnaughtan⁹⁶⁾

Table 6. Pressure effect on discontinuities in Arrhenius plots of membrane bound enzymes

Enzyme	dT/dp	Ref.
Nitrogenase (Azotobacter)	20	94
NaK-ATPase (pig kidney)	27.7	95
NaK-ATPase (A. Laidlawii)	16	96
Ca-ATPase (Sarcoplasmic ret.)	27	97
Phospholipids	17-22	Table 5

have also observed shifts in the breaks of a membrane bound ATPase.

The sarcoplasmic reticulum CaMg ATPase is also interesting especially since the work of Dean and Tanford⁹⁸ who ascribe the break to the protein. Heremans and Wuytack⁹⁷ have measured the activity optically with a coupled enzyme assay up to 1000 atm. At 25°C a break in the log activity *versus* pressure is observed at 300 atm. At higher temperatures this break shifts to higher pressure by 27°C/1000 atm. We conclude that if pressure and temperature affect the boundary lipids, then these lipids show a normal melting behaviour although they are supposed to interact strongly with the protein.

A more general conclusion from this work is that temperature together with pressure is an important parameter for the study of membrane phenomena. More specifically because thermotropic lipid transitions are more sensitive to pressure changes than protein conformational changes are, pressure changes present a useful tool for discrimination between both phenomena in more complex systems such as the heat activation of fungal spores. Pressure has a small effect on fungal spore heat activation⁹⁹ implying a protein conformational change as the triggering mechanism of the heat activation. In view of the high carbohydrate content of these spores, changes in the conformation of polysaccharides are however not excluded. These transitions also show a small pressure effect.⁹ The pressure dependence of glass transitions temperatures in hydrogen bonded molecular liquids is also small.¹⁰⁰ Hydrogen bonding might therefore also explain the small pressure effect on proteins.

7. Prospects for the Future

"Of the various thermodynamic properties of a system, volume and area appear the easiest to grasp intuitively, and an attempt is made to extend these macroscopic concepts to the molecular level".

This statement¹⁰¹ makes very clear what will be the main theme for the future. The dynamic nature of proteins as revealed by the freedom of rotation of groups inside the protein; the response of the protein structure to the binding of small molecules; the interaction of protein molecules to form supramolecular structures such as microtubuli; their interaction with nucleic acids to form ribosomes and chromatin and finally their interaction with phos-

pholipids to form biomembranes, are all examples of the way nature exploits molecular interactions to obtain macroscopic results, *i. e.* life.

It is the task of those who work with high pressure techniques in the field of biophysical chemistry, to characterize these processes in terms of volume changes.

In this review we have concentrated on systems at equilibrium. With the availability of kinetic techniques such as stopped flow and NMR, studies of the dynamics of protein folding and ligand binding are now possible.

Our present understanding of the volumetric behaviour of proteins will change. There is a good chance that nature has some surprises in store for us.

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Department of Chemistry
Katholieke Universiteit Leuven
Clestinienlaan 200D
B-3030 Heverlee
Belgium