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Contents

Molecular Mechanisms on Carbonate, Phosphate, and Silica Deposition in the Living Cell

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Contents

Fool Canst tell how on oyster makes his shell?
Lear No.
Fool Nor I neither; but I can tell why a snail has a house.
Lear Why?
Fool Why, to put's head in.
(William Shakespeare: King Lear, Act I; Scene V)

I. Introduction

Mineral deposition in biological systems can be beneficial to an organism and lead to the formation of bones, teeth and shell material, or result in the accumulation of essential elements such as calcium and phosphorus. There is also a pathologic aspect in biomineralization, for example, thickening along the inner portion of the artery wall – atherosclerosis[1–3], and stone formation in the urinary tract[4,5]. A related field is mineral decay, *e.g.* caries formation or resorption of bone material[6]. In short calcification falls into two categories – the normal and the pathologic one.

Focal point of this article is the molecular interaction between metal ions and skeletal tissues in "healthy" cells. Special attention is given phenomena leading to

	Bacteria	Algae	Protozoa	Porifera	Coelenterata	Bryozoa	Brachiopoda	Sipunculida	Annelida	Mollusca	Arthropoda	Echinodermata	Chordata
Carbonates													
Calcite	?	+	+	+	+	+	+		+	+	+	+	+
Aragonite	+	+	+	+	+	+		+	+	+	+		+
Vaterite		?											?
$CaCO_3$ monohydrate													+
"Amorphous"					+				+	+			+
Phosphates													
Dahllite										+	?		+
Francolite							+			+			+
"Amorphous"										+			
Silica													
Opal		+	+	+						+			
Fe-Oxides													
Magnetite										+			
Goethite										+			
Lepidocrocite				+						+			
"Amorphous" hydrates			+							+			
Sulfates													
Celestite			+										
Barite			+										
Gypsum					+								
Halides													
Fluorite										+			
Oxalates													
Weddellite										+			

Fig. 1. Distribution of skeletal minerals according to phylum (after Lowenstam[8])

the nucleation of a crystal seed and its subsequent oriented growth (epitaxis) in the direction of an organized structure.

About twenty different skeletal minerals are reported from organisms[7, 8], however, only four are common: (1) aragonite, (2) calcite, (3) dahllite = carbonate hydroxyapatite, and (4) opal. The remaining minerals depicted in Fig. 1 are either trace constituents or occur only in a few isolated species. It is for this reason that the article concentrates on carbonate, phosphate and silica deposition in plants and animals. For reviews on general aspects of biomineralization and discussions on individual taxonomic classes see Ref.[9–47].

Looking at the literature in the field of biomineralization, one notices, that the majority of articles is descriptive in nature. On the basis of electron micrographs or thin section studies, the intricate relationships between mineral phase and organic matrix are investigated. Other papers deal with the chemical composition of the mineralized tissue and the minerals. Only a few authors address themselves to the question of metal ion transport mechanisms in cellular systems and the solid state principles involved in mineral deposition on organic substrates. All three sets of information, however, are essential to understand calcification processes. It appears, therefore, that information on the functionality of metal ions in living systems and their role in mineral deposition are particularly desired in this area of research.

II. Metal Ion Coordination

Metal ions are a key factor in the structural organization of biochemical molecules and the functional processes operating in the genetic and metabolic apparatus. They form coordination bonds with oxygen and nitrogen and introduce a higher structural order in participating macromolecules[48].

To illustrate the type of structures that will arise when metal ions interact with organic compounds, three examples which reveal the basic details of metal ion coordination complexes, are given below:

(i) EDTA

Ethylene diamine tetra-acetic acid (EDTA) is a common chelating agent. It is able to pick up polyvalent metals in its "clawlike" structure. Metal cations can become isolated from their solvent medium by such a complex. The structure of the EDTA complex having manganese as a central cation[49] is shown in Fig. 2. Coordination between Mn^{2+} and EDTA yields a complex which is composed of Mn $(N_2O_5)^{2-}$ sphere-like assemblies having a diameter of approximately 6 to 6.5 Å. Based on X-ray data, a coordination polyhedron can be constructed (Fig. 2), which describes the crystalchemical relationships exhibited by the $(Mn^{2+}(EDTA)^{2-})$ chelate.

In general, the concept of metal ion coordination polyhedra is a "molecular shorthand" which condenses real molecular assemblies into a simpler structural form. As such, it is a convenient tool to describe molecular entities.

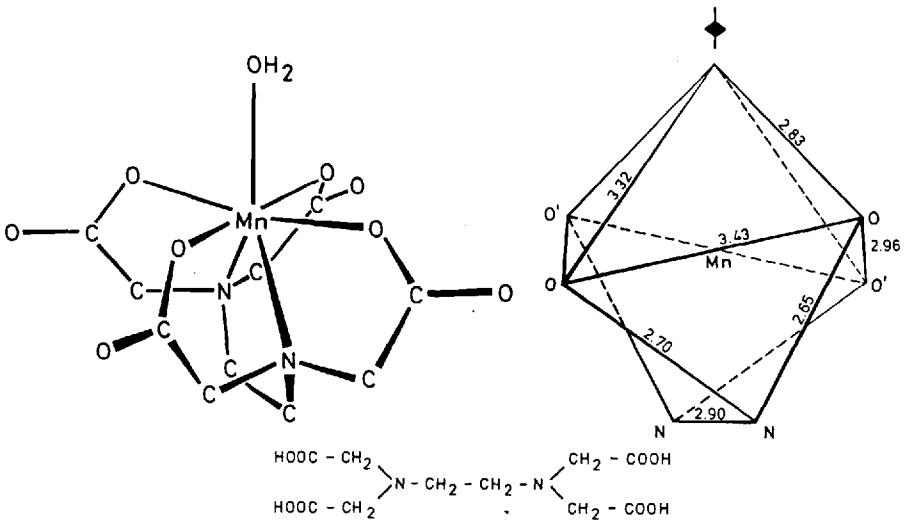


Fig. 2. Coordination complex of EDTA and Mn^{2+}: Mn $(EDTA)^{2-}(OH)_2$ (left). The coordination polyhedron: AB_7 of the chelate (right). The numbers refer to the length of the edges in Å units (after Richards *et al.*[49])

(ii) DNA

A two-dimensional model of deoxyribonucleic acid (DNA) in Na^{1+}coordination (Fig. 3) shows the spacial dimensions for PO_4 tetrahedra and $Na^{+}O_6$ octahedra[48]. The polyhedra determine the shape of the macromolecules and are instrumental in forcing two single stranded DNA's into a double helix. The base-base hydrogen bonds cannot account for the number of stereotypes that exist. Metal ion coordination also inhibits the folding of a single stranded DNA; folding of a single stranded DNA and generation of branched structures takes place only when metal ions are removed from the system.

Different metal ions can occupy the position of the sodium ion. Magnesium was found to be an effective ion in stabilizing the helical regions of RNA and DNA[50–52]. The presence of coordinated metal ions and PO_4 gives rise to a well-defined three dimensional network which is utilized by enzyme systems of the protein synthesis apparatus. It is of interest that the type of metal ion determines the helical arrangement. Whereas sodium generates double stranded helices, the addition of a small number of divalent metals causes the formation of a triple-stranded molecule[53–55].

(iii) Phospholipid Membranes

A cross-section of a phospholipid membrane-surface is depicted in Fig. 4; omitted are proteins and polysaccharides to better reveal the structural interrelationships.

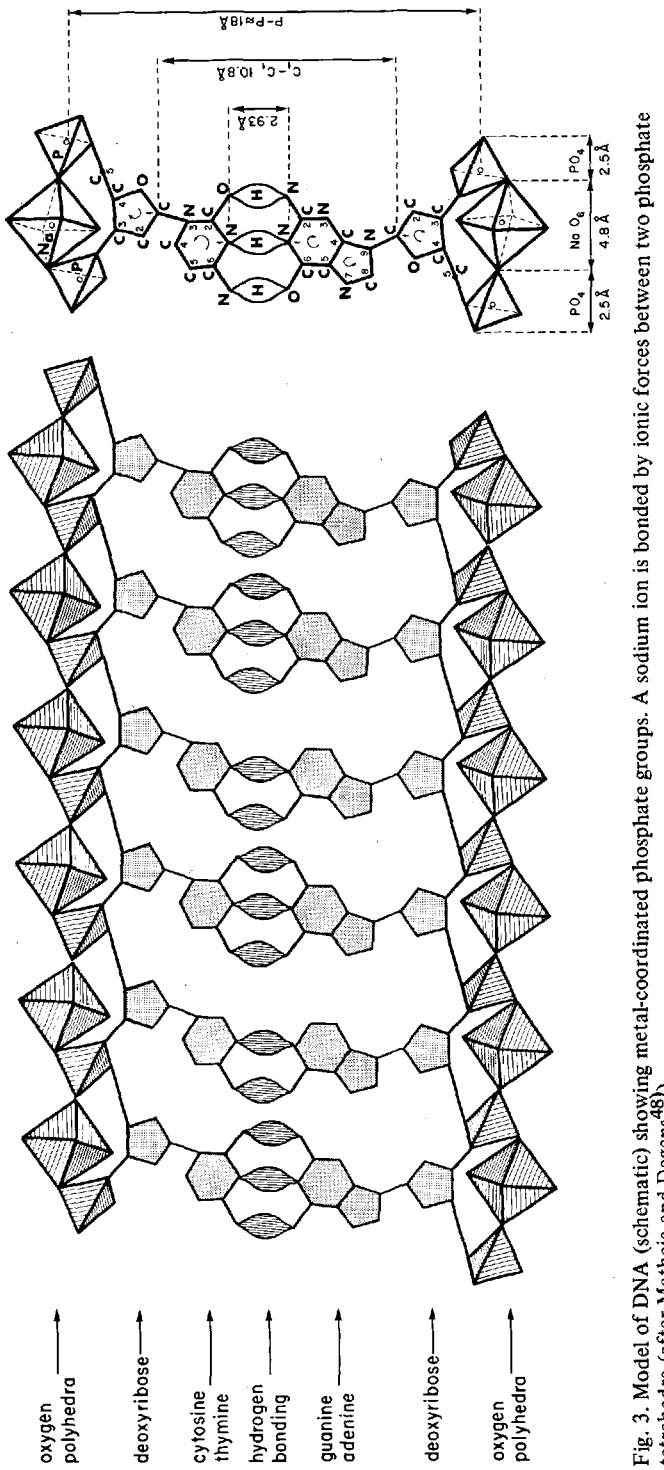


Fig. 3. Model of DNA (schematic) showing metal-coordinated phosphate groups. A sodium ion is bonded by ionic forces between two phosphate tetrahedra (after Matheja and Degens[48])

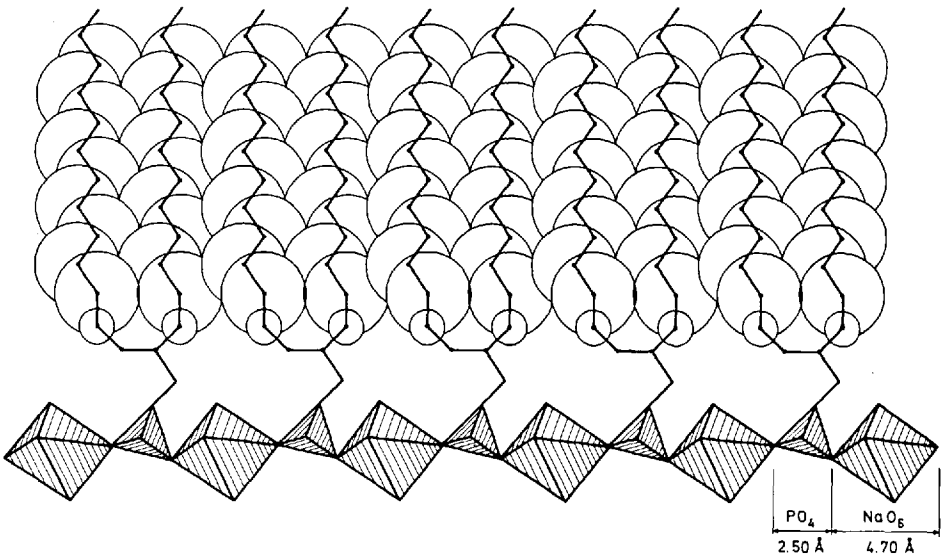


Fig. 4. Schematic phospholipid membrane cross-section composed of double aggregates of hydrocarbons and oxygen polyhedra. Glycerol is presented schematically; the PO_4-residues have been omitted. The molecular dimensions are based on the Na^+O_6 octahedron (after Matheja and Degens[48])

Glycerol is only shown schematically. The surface exhibits a lipid-oxygen assemblage and shows the space requirements for an oxygen coordinated metal polyhedron and for hydrocarbon chains.

Theoretical consideration on the type of ionic forces established between Me^{n+} and PO_4^{n-} units agree with analytical data in molecular surface research[56, 57].

A statistical thermodynamic analysis indicates that a pure lecithin molecule in water will aggregate to bilayers with a maximum disorder due to uncompensated charges[58]. They represent unstable fabrics, where the thickness of the bilayers varies with the amount of incorporated water.

When metals are available, the laminae are stabilized by bonding forces as shown in studies on phospholipid-water systems[59]. Even films of myristic acid and metal ions are solid up to a cross-section area of 50 Å per molecule[60, 61]. The numerous phase transitions of pure phospholipid-water systems[62] and the polymorphism of phospholipid crystals[63] suggest that even pure phospholipid bilayers – in their true nature – do not represent stable lattice structures but require metal ions for coordinative purposes.

The molecular geometry of phospholipid membranes is thus structurally analogous to inorganic phosphate minerals in that corrugated layers of metal ion-phosphate coordination complexes exist in membranes and minerals. In Fig. 5, the structure of a typical phosphate mineral is shown to reveal the type of molecular pattern that is exposed at the ionic surface of phospholipid membranes[64, 65]. For a recent review on membrane structure see[66].

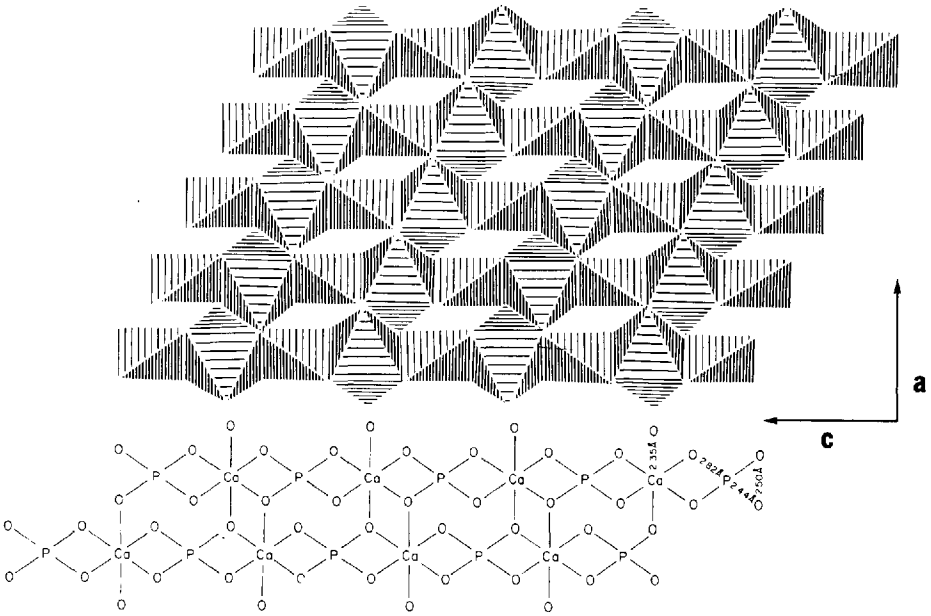


Fig. 5. Presentation of molecular structure of a sheet composed of phosphate-cation chains cross-linked in a planar pattern. Such structures are known from calcium monohydrogen phosphate dihydrate $CaHPO_4 \cdot 2\,H_2O$ and from monohydrate $CaHPO_4 \cdot H_2O$ (after MacLennan and Beevers[65]; Beevers[64])

In conclusion, metal ions are an integral part of various biological macromolecules. Stripping or substitution of metal ions will introduce conformation and coordination changes. Consequently, the association of metals and organic molecules in the form of metal ion coordination complexes is an ordinary feature of the living cell and nothing unusual. In turn, biomineralization represents only an outgrowth of these characteristics.

III. Epitaxis

The growth of crystalline material on other crystal surface is a well studied subject in the field of crystallography. The process is commonly described under the heading "epitaxis" a term which is derived from the Greek ταασειν meaning: to arrange or to organize. Epitaxis, however, can also proceed on organic templates with the resultant formation of biominerals. The following discussion is given to define the principle mechanisms of epitaxis.

Epitaxis on solid state phases should be viewed in relation to catalysis because both processes follow a similar reaction path. In Fig. 6, the main steps are schematically outlined. Based on data on the molecular configuration of catalytic processes

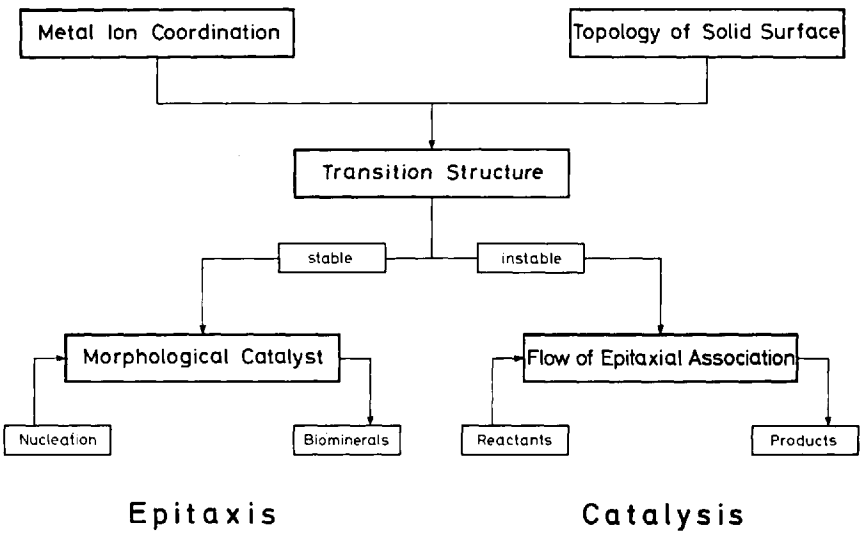


Fig. 6. Reaction scheme of epitaxis and catalysis

on solid state substrates it can be shown, that catalysis represents a process by which a solid state surface "tries" to establish a thermodynamically favorable phase transition structure with the adsorbent. The crystal habitus is catalyzed through solution participants by the formation of boundary structures[67–72]. The specific topology introduced by bonding forces of a solid state surface results in specific phase transition structures[73–82]. When these phase transition structures are chemically stable, the result is epitaxial coordination, or oriented intergrowth[83–88]. In contrast, should the building of these transition structures cause a chemical alteration of the adsorbent, such as polymerization, or hydrogenation and dehydrogenation, the problem becomes one of catalytic processes. The principles of catalysis as biologically exercised by enzymes are in accordance with observation on inorganic systems[89–91].

The decision whether we are dealing with an epitaxial or catalytic association is thus structurally determined. Catalysis represents a flow of epitaxial associations, whereas epitaxis is dependent on a "frozen-in" transition structure, *i.e.* the presence of a morphological catalyst. Adsorbent and solid state substrate become a new structural entity. Only removal or stripping of the adsorbent will reactivate the morphological catalyst.

Organic matrices with a capactiy to act as morphological catalyst in the deposition of minerals must consequently be crystalline in nature. In the literature, colloidal or amorphous phases are frequently postulated as organic starting materials in biomineralization. Such a viewpoint, however, is not consistent with oriented epitaxial growth.

In the following, three crucial experiments are described which will show the validity of the crystalline model.

(i) Thermal Degradation of Calcified Tissue

Proteins and glycoproteins are the principal constituents of bone and shell organic material. It was, therefore, of interest to investigate their response to a heat treatment at elevated temperatures. In case such a protein is just intercalated with the minerals as a separate phase, its behavior during thermal decomposition should be almost identical to that of common proteins such as silk, hair or wool; such proteins will readily decompose at temperatures between 100° and 200 °C. In contrast, are minerals and proteins chemically "fused" together by some means, thermal degradation should proceed at much higher temperatures. This is so, because protein and mineral phases are incorporated into the same lattice complex. Under such circumstances chemical changes introduced during thermal degradation can only take place at the moment the crystalline phase boundary is destroyed.

To test this supposition the shell of *Nautilus pompilius* (mollusc) containing almost 3% organic matter was subjected to pyrolysis at temperatures of up to 500 °C for periods of several hours and days. The experiments were performed under dry conditions and in the presence of distilled water. Some of the data are compiled in Table 1.

At 150 °C, the dry runs showed no significant changes in the relative and absolute amounts of amino acids. The wet runs, however, exhibited distinct changes and some of the relationships are depicted in Fig. 7. Noteworthy is the stability of aspartic acid, glutamic acid and alanine, and the instability of threonine and serine. One can conclude: (1) organic matter and mineral represent distinct organic-inorganic complex since aragonite and proteins remain intact; (2) hydrolytic processes will preferentially release amino acids and small peptides less intimately tied to the mineral surface, and (3) wet combustion will liberate glutamic acid as a peptide.

(ii) Aragonite – Calcite Transformation

At 25 °C and one atmosphere pressure the values for calcite → aragonite are[92]:

Heat change $\Delta H°$	$= 40$ cal mole^{-1}
Entropy change $\Delta S°$	$= -1$ cal mole^{-1} deg^{-1}
Free energy change $\Delta G°$	$= +250$ cal mole^{-1}
Molar volume change $\Delta V°$	$= -2.78$ cm^3 mole^{-1}

These values indicate that calcite is more stable than aragonite and that with increase in temperature aragonite should alter into calcite. Under dry conditions, rates are slow below 100 °C and complete transformation will take millions of years. In the presence of water, however, the aragonite → calcite transformation takes only a few hours in the temperatures regime between 50° and 100 °C[93, 94].

Nautilus pompilius deposits a shell composed entirely of aragonite. Experiments[95–97] conducted at temperatures between 150° and 900 °C showed the shell aragonite to be stable for a few hours at temperatures of up to 300 °C even under aqueous conditions. Above 300 °C a rapid transformation into calcite was observed

Table 1. Heating experiments on shell material of *Nautilus pompilius* at temperature of 150° to 250 °C under dry conditions and in the presence of water. Amino acids in residues/1000; oxygen and carbon isotopes expressed as δ-deviation relative to a belemnite standard

		$CaCO_3$ (150 °C)								H_2O leachings (150 °C)						$CaCO_3$ (250°)
		Dry				Wet				Free				Total (6 N HCl)		Wet
Amino acid	original	48[1])	96	168	216	24	48	120	216	24	64	120	216	64	96	2
Aspartic acid	80	81	81	77	78	105	103	131	140	44	31	22	16	43	25	136
Threonine	21	21	20	22	22	12	13	10	5	4	1	1	1	5	3	8
Serine	96	95	93	96	96	55	45	38	17	108	69	52	42	52	28	38
Glutamic acid	43	43	44	43	46	65	75	78	80	tr.	tr.	tr.	tr.	43	54	85
Proline	13	11	11	11	12	7	7	12	16	4	3	3	4	12	8	22
Glycine	347	347	342	345	349	255	233	224	229	600	625	607	560	463	496	233
Alanine	237	238	242	246	241	286	333	360	360	175	203	247	287	222	257	242
Cystine (half)	1	1	1	1	1	3	2	4	3	5	2	1	1	1	2	1
Valine	14	15	14	13	14	22	27	20	24	9	8	7	11	18	17	54
Methionine	1	1	1	1	1	1	1	1	1	9	3	1	1	1	1	1
Isoleucine	12	12	11	12	12	18	18	15	13	7	7	6	9	18	11	12
Leucine	19	20	19	20	20	30	29	28	29	15	14	12	17	27	26	33
Tyrosine	5	6	6	5	5	2	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	5
Phenylalanine	51	50	55	50	50	52	48	47	41	7	31	38	52	78	69	68
Lysine	4	4	4	4	3	8	9	6	12	2	1	tr.	tr.	tr.	tr.	4
Histidine	2	2	2	2	3	2	2	1	2	tr.	tr.	tr.	tr.	tr.	tr.	3
Arginine	50	50	49	49	44	73	43	13	15	tr.	tr.	tr.	tr.	4	tr.	43
$\delta\ ^{18}O$	+1.29	+1.27	+0.94	+1.02	+0.95	−0.17	−0.52	−0.29	−0.50							
$\delta\ ^{13}C$	+1.42	+2.12	+2.57	+3.15	+2.32	+2.38	+3.53	+2.37	+2.57							

[1]) Reaction time in hours.

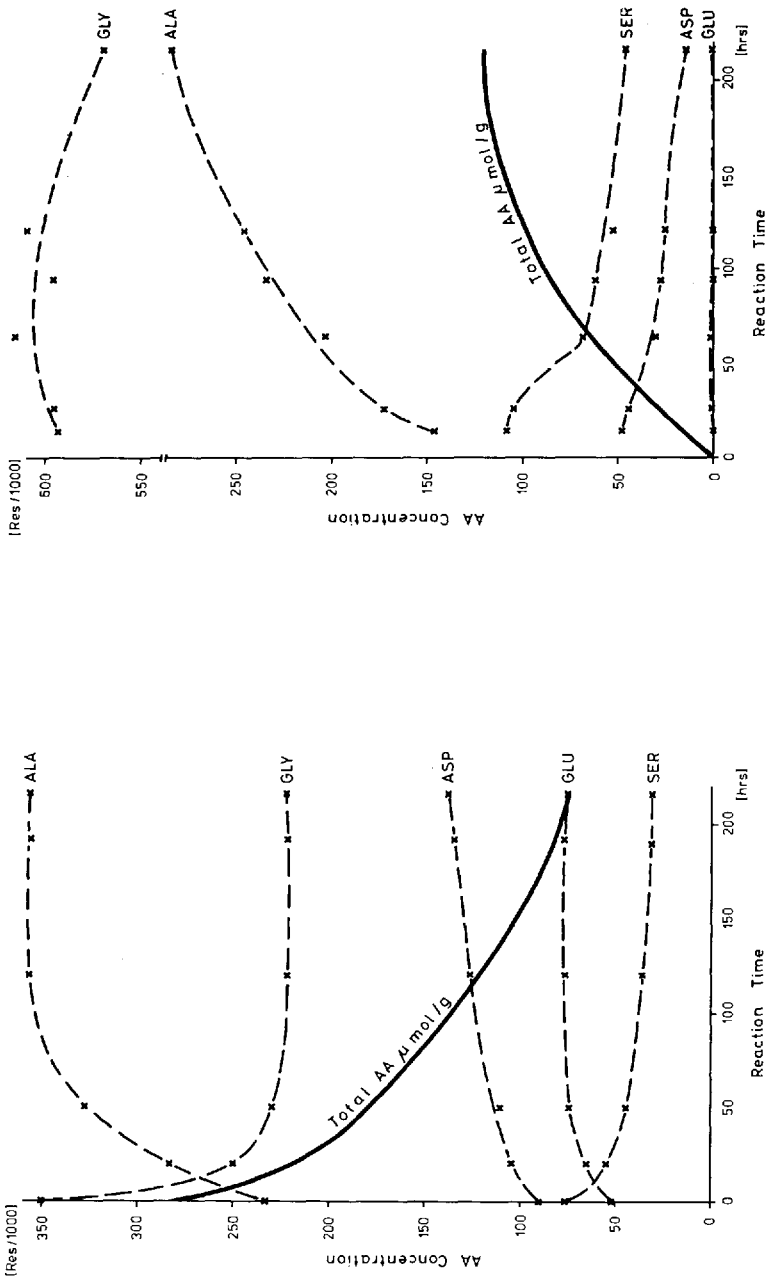


Fig. 7. Thermal decomposition rate of shell organic matrix (*Nautilus pompilius*) at 150 °C in the presence of water. Individual amino acids are reported in residues/1000 and total amino acid concentration in μmol/g; numbers on the ordinate are residues/1000 and μmol/g, respectively. Graph on the left is hydrolyzed fraction of total shell material after thermal treatment; and graph on the right the unhydrolyzed fraction in the water

and the ultrastructural relationship revealed a complete recrystallisation of aragonite into calcite. It is of interest that in wet runs, isotope exchange between water and mineral is quite sluggish which again underscores the stability of the aragonite – protein complex (Table 1).

The kinetics of recrystallisation of aragonite to calcite has been studied at a temperature of 66 °C in the presence of inhibiting and accelerating compounds[98]. The results are given in Fig. 8. In distilled water pure aragonite completely recrystallizes into calcite in less than 24 hours. Basic and neutral amino acids accelerate the transformation, whereas glutamic and aspartic acids strongly inhibit the reaction. This phenomena can best be explained in terms of catalysis and epitaxis, respectively.

That is, basic and neutral amino acids follow the pattern long recognized for strongly dissociated solutions which promote transformation, whereas acidic amino acids form a protective overgrowth on the aragonite surface via carboxyl groups.

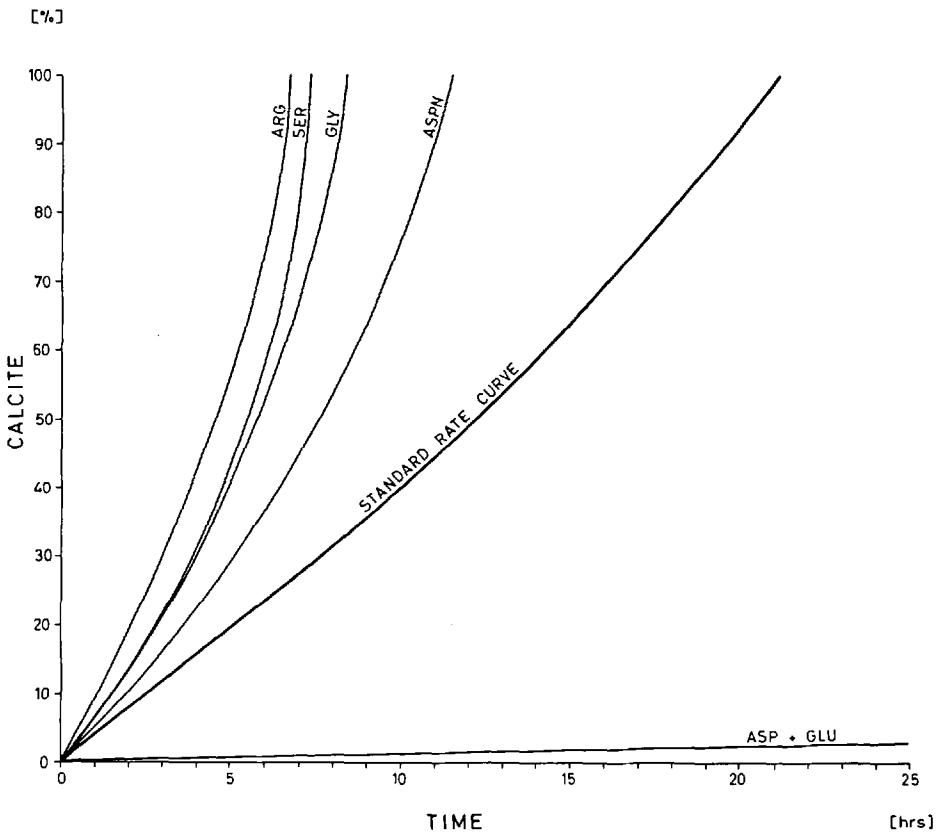


Fig. 8. The effects of basic, neutral and acidic amino acids on the rate of recrystallization of aragonite to calcite; the standard rate curve is given for comparison (after Jackson and Bischoff[98])

(iii) Amino Acid – Mineral Interaction

Ultrastructural patterns that arise when amino acids or small peptides interact with mineral surfaces have been studied in some detail[99, 100]. A poly-L-alanine solution evaporated at 40 °C on a rhombohedral plane of R-quartz deposits the peptide principally in α-conformation. Chain-folded helices are aligned in the form of lamellae which exhibit a sharp phase boundary at the organic-mineral contact zone (Fig. 9). Frequently the lamellae are split along the direction of their fold axis ("zipper effect"). Insertion of β-pleated sheets running perpendicular to the long axis of the lamellae act as dispersion forces and cause the formation of cross-β-

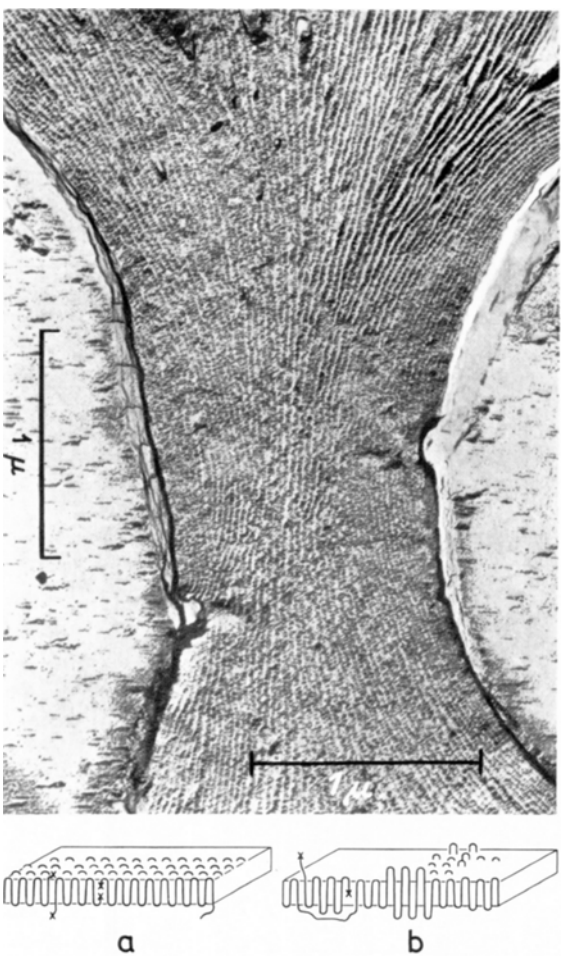
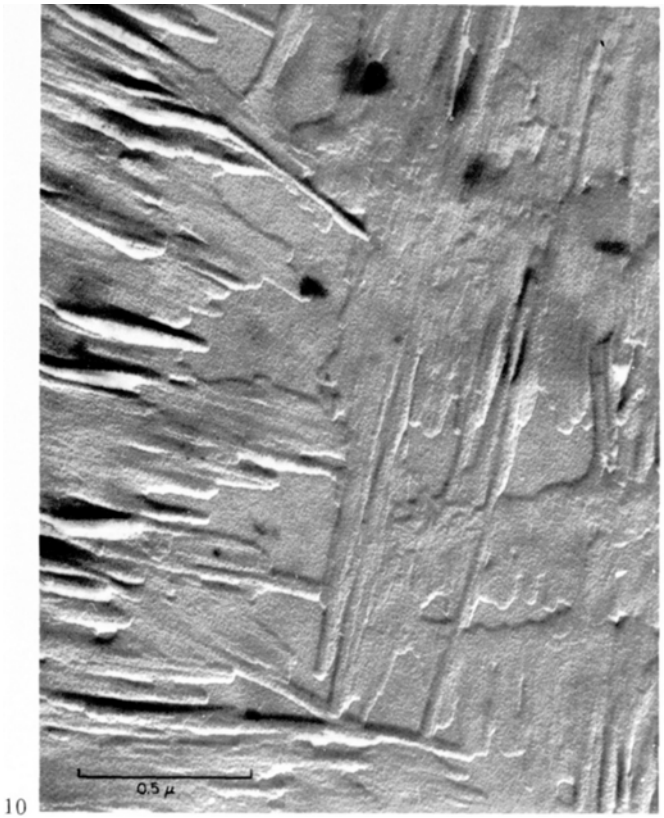


Fig. 9. Electronmicrograph (Pt/C replica) showing epitaxial association of poly-L-alanine and R-quartz. Note the lamellar pattern and the insertion of new lamellae due to dispersion bonding forces. A smooth chain-folded surface, as shown in (a) is developed. In contrast a rough-chain-folded surface (b) is developed in Fig. 10. Start and termination of a peptide chain is indicated in (a) and (b) by an x (after Seifert[99, 100])



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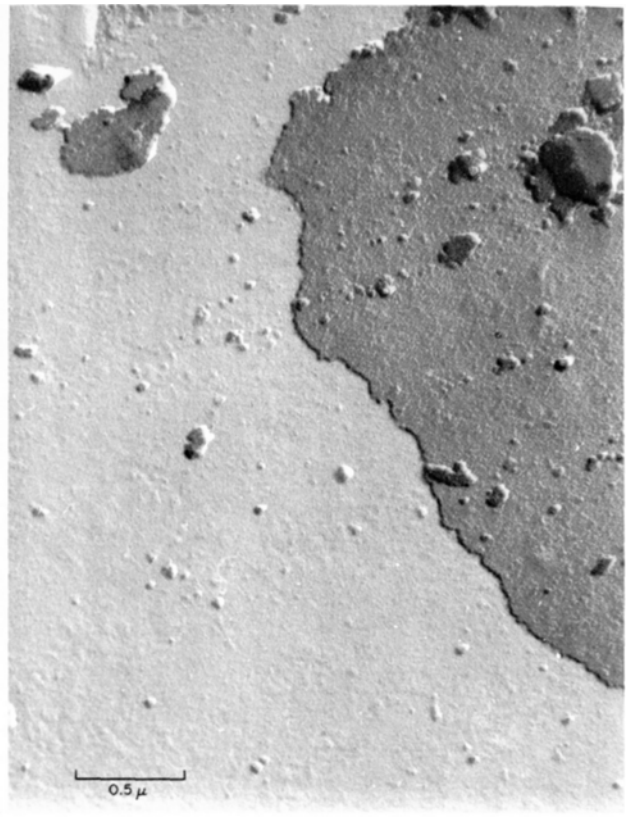

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Fig. 10. Electronmicrograph (Pt/C replica) showing epitaxial association of L-alanine and quartz. Chains are oriented in two preferred directions and a rough chain-folded surface is developed. See graph (b) Fig. 9

Fig. 11. Electronmicrograph (Pt/C replica) showing epitaxial association of L-aspartic acid and quartz. A smooth layer of aspartic acid is developed.

structures, as indicated by IR-spectra. It is of note that increase in temperature during evaporation of the peptide solution will progressively favor β-over α-conformation.

L-alanine deposited on a rhombohedral surface of quartz exhibits two preferential directions. A rough chain-folded surface is developed which is a result of multiple nucleations (Fig. 10).

In contrast, aspartic acid produces a rather smooth layer (Fig. 11). The fact that a complete recovery of amino acids requires a 6 N HCl hydrolysis suggests that at least a partial polymerization occurs. Related studies on a number of minerals has indeed shown the polymerization potential of solid state surfaces. Thus, a mineral surface may simultaneously act as a template and a catalyst[101, 102].

The different ultrastructural patterns obtained for alanine and aspartic acid are a result of the ability of alanine to become adsorbed to the mineral substrate by means of hydrogen bonds, whereas aspartic acid is principally bound via carboxyl groups.

Under the assumption that poly-aspartic acid is generated, the carbonyl groups should be aligned in a hexagonal array for sterical reasons, whereby each corner of the hexagon is occupied by one oxygen. Such an arrangement represents a rather stable configuration and may explain why aspartic acid inhibits the aragonite-calcite transformation process[98].

In nature, a series of minerals are known to interact with organic substances, notably carbohydrates and amino compounds[103–108]. In the context of this discussion the dominance of acidic amino acids in oolites is significant[109–111]. Oolites are spherical bodies up to 1–2 mm in diameter which are chemically precipitated in tropical lagoons, *e.g.* Bahamas, Bermudas and Red Sea in the form of aragonite and accumulate as fine sand. The process of precipitation is a slow one and may proceed over a period of a few hundred years.

The outstanding trends are: (1) low concentration of basic amino acids (2) predominance of acidic amino acids (50 to 70% of total), (3) stepwise increase – from outer layer to inner core – in aspartic acid with a concomitant decrease in the leucines, serine, threonine and alanine, (4) uniform glycine and glutamic acid values, and (5) low hexosamine content. The high abudance of aspartic acid in oolites suggests that this amino acid plays a key role for Ca^{2+} fixation. The lack of basic amino acids and the gradual loss of others indicate that these amino acids are structurally removed from aspartic acid-induced nucleation centers and, therefore, are not strongly bound to aragonite crystals. Glutamic acid and glycine may be situated in close proximity of an aspartic acid nucleation site and, therefore, be protected from degradation. The stepwise changes in concentration (Fig. 12) suggest that oolites grow intermittently as opposed to continuously as is frequently assumed.

The source of amino acids in the oolites is unknown. The systematic distribution and the preference for acidic amino acids suggest a selective extraction from dissolved organic matter generated by the local biota. The adsorbed amino acids form a thin organic coat around the oolite and act as a template for the nucleation and oriented growth of aragonite crystals. Several organic layers can be recognized in thin sections.

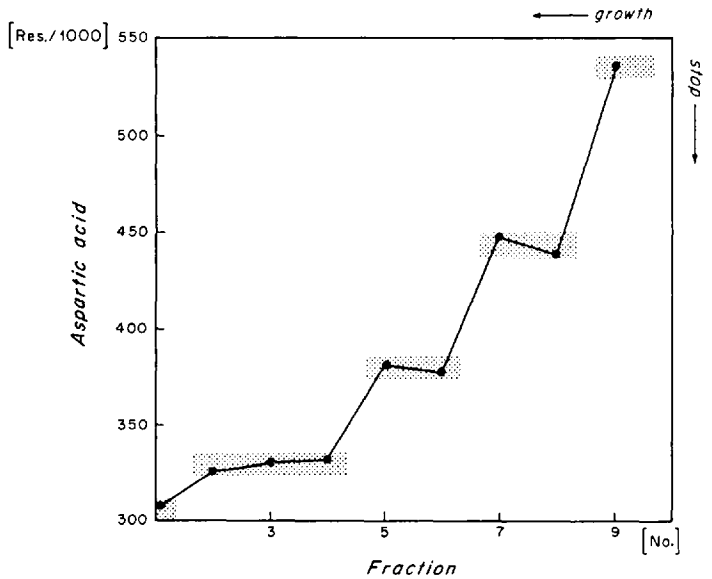


Fig. 12. Changes in aspartic acid concentration in consecutive HCl etchings of a 0.5 mm oolite fraction. Sample No. 1 represents the outer portion of the oolites and sample No. 9 represents the inner core
^{14}C analysis (W. Broecker, pers. comm.) indicates an age of *ca.* 2000 years for the inner core of the oolites. The steps indicate that oolites grow intermittently

In conclusion thermal degradation studies on *Nautilus pompilius* indicate that mineralizing matrix and aragonite shell represent a true structural entity. By the sharing of oxygens in protein and mineral lattices we will generate phase boundaries of the type that are present, for instance, in the common clay mineral kaolinite. Here, aluminum octahedra and silica tetrahedra incorporate the same oxygens and hydroxyls, and layers composed of octahedra and tetrahedra arise (Fig. 13).

Acidic amino acids seem to play a key role in: (1) the fixation of calcium, (2) the nucleation of $CaCO_3$ crystals, and (3) the oriented growth of the mineral phase. There appears to be no essential difference between biochemical or geochemical template-induced mineral formation. The only requirement is the presence of a calcium-specific template and an environment suitable for the deposition of calcite or aragonite.

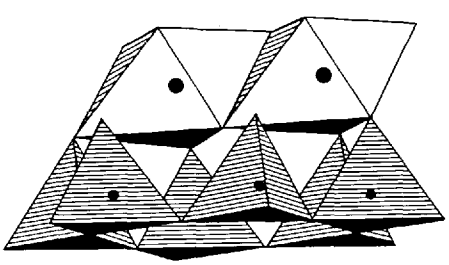


Fig. 13. Arrangement and interrelationships between silica tetrahedra and aluminum octahedra in a common clay mineral, kaolinite

IV. Mineral Equilibria

The two polymorphs of $CaCO_3$, *i.e.* the rhombohedral calcite (space group: $R\bar{3}c$) and orthorhombic aragonite (space group: *Pmcn*) represent the bulk of the carbonates encountered in biological systems.

Of the four prominent pure end-member phosphate species of the apatite group only dahllite (space group: $C6_3/m$) is a common biomineral[28]. This carbonate apatite is a distinct variety of apatite rather than a mixture of $CaCO_3$ and hydroxyapatite. Expressed as oxides, the mineral content of bovine cortical bone (dry; fat-free material) has the following chemical composition[112]:

CaO	MgO	Na_2O	K_2O	SrO	P_2O_5	CO_2	F	Cl
37.56	0.72	0.99	0.07	0.04	28.58	3.48	0.07	0.08

Silica is present in organisms in the form of opal ($SiO_2 \cdot nH_2O$; amorphous). The amount of water may vary considerably.

In the following a few pertinent data on mineral equilibria in carbonate, phosphate and silica systems are summarized. The subject has been extensively reviewed elsewhere[113].

(i) Carbonate

The relationship in the sea water carbonate system has been thoroughly investigated, *e.g.*[114–119]. Surface sea water is commonly supersaturated with respect to aragonite and calcite. This disequilibrium state is attributed to the presence of a series of inhibitors, most notably magnesium and dissolved organic matter that interfere with the nucleation of a $CaCO_3$ crystal seed[120]. It has been suggested that inorganic precipitation of $CaCO_3$ in average sea water would require about 10^5 years[121].

Increase in water depth will shift the equilibrium and between 3.000 and 4.000 m the ocean starts to become unsaturated with respect to aragonite and calcite due to pressure effects[122–124]. In contrast, the majority of river and lake waters are unsaturated at all depths, because precipitation of $CaCO_3$ will readily take place the moment saturation is exceeded. The solubility products are[92]:

$$[Ca^{2+}]\,[CO_3^{2-}] = 4.7 \times 10^{-9} \text{ (calcite)}$$
$$= 6.9 \times 10^{-9} \text{ (aragonite)}$$

which again shows that aragonite is less stable than calcite.

In the presence of organic matter, inorganic precipitation of $CaCO_3$ is inhibited or accelerated depending on the nature of the dissolved constituents[125–128].

Of special significance to calcification processes is the pronounced effect of temperature and pH on the concentration and relative proportion of the carbonic acid substances in natural waters. At 0 °C, the solubility of carbon dioxide in sea water is about 2.5 times greater than at a temperature of 30 °C and the P_{CO_2} of sea

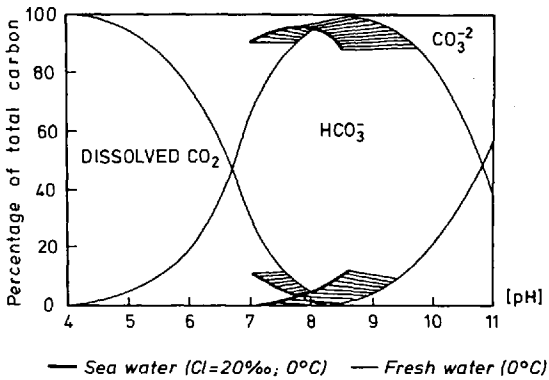


Fig. 14. Distribution of forms of inorganic carbon in fresh and sea water, as percent of total present. "Dissolved CO_2" is the sum of the carbonic acid (H_2CO_3) and "molecular CO_2" present (after Skirrow[114])

water changes 1 per cent per °C. The relationship between pH and the ratio of the various dissolved carbonate species is shown in Fig. 14[114].

(ii) Phosphate

In sea water, we encounter one phosphate group for every 10^6 water molecules. This relationship varies slightly because of the vertical fluctuation in the phosphorus content in the sea. For saline water systems, data on the solubility product of a calcium phosphate is difficult to obtain inasmuch as – aside from temperature and hydrostatic pressure – the solubility product is dependent on the type and amount of solutes present. The apparent dissociation constants of H_3PO_4 are defined by the equations[129, 130]:

$$K_1' = \frac{a^*[H_2PO_4^-]}{[H_3PO_4]}; \quad K_2' = \frac{a^*[HPO_4^{2-}]}{[H_2PO_4^-]}; \quad K_3' = \frac{a^*[PO_4^{3-}]}{[HPO_4^{2-}]}$$

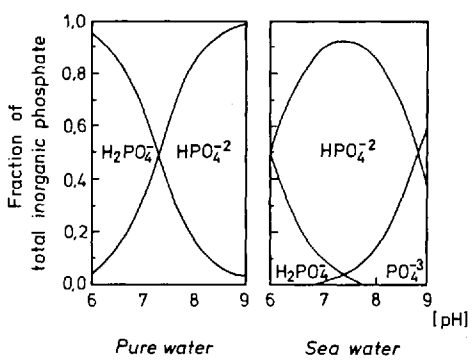


Fig. 15. Distribution of phosphate species at 20 °C (after Kester and Pytkowicz[130])

The brackets indicate the molar concentrations of the various molecular species. The empirical quantity a^* is defined by $pH = -\log a^*$. In sea water, pH measurements do not yield a thermodynamic hydrogen ion activity due to liquid junction and asymmetry potentials; a^* only approximates the hydrogen activity a_{H^+}. For sea water of 33‰ salinity at 20 °C and at pH 8, 87% of the inorganic phosphate exist as HPO_4^{2-}, 12% as PO_4^{3-}, and 1% as $H_2PO_4^-$. Of the PO_4^{3-} species 99.6% is complexed with cations other than Na^+. The equilibrium relationship for the system is shown in Fig. 15.

No reliable estimates presently exist on the degree of saturation of sea water with respect to calcium phosphate. The apparent solubility product reported in the literature[131]:

$$[Ca^{2+}]^3_{sat}\,[PO_4^{3-}]^2_{sat} = 3.73 \times 10^{-25}$$

has a probable error of at least three orders of magnitude; this is so, because it was not known at that time that orthophosphate is present in sea water in a predominantly complexed form.

Solubility calculations are commonly made relative to apatite as the solid phase[132, 133]. Although the values may not be to far off from the correct ones we should remember that carbonate apatite is the principal phosphate mineral deposited in sedimentary environments and biological systems as well[28, 134]. On that basis and the fact that phosphorites do form today the ocean is close to saturation, whereas the phosphorus content in the majority of rivers and lakes is generally low and below saturation..This situation may soon change as a consequence of the increasing discharge of phosphates into the freshwater environment by households, industries and agriculture.

(iii) Silica

The lowest free state of silica at room temperature is quartz and accordingly, all other modifications are metastable. However, to precipitate quartz, activitation

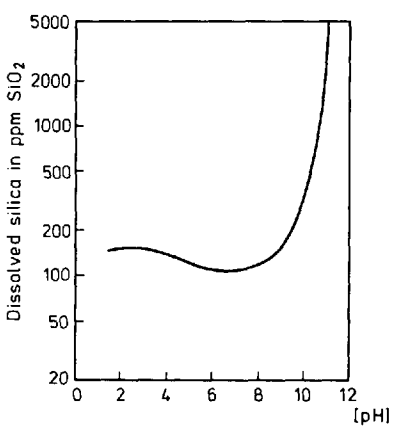


Fig. 16. Solubility (saturation concentration) of oligomeric silicic acid at different pH values. (After Alexander *et al.*[140]; Okamoto *et al.*[139]; Krauskopf[138])

energies of the order 200 kcal/mole are required. Consequently the first substance that precipitates from aqueous solutions of silicic acid is amorphous silica of various degrees of hydration[135, 136]. Reorganization into crystalline phases (microcrystalline quartz) proceeds extremely slowly[137].

To deposit amorphous silica at room temperature saturation concentration of about 100 to 150 ppm dissolved silica must be reached[138–140]. These fluctuations are due to slight pH effects on solubility (Fig. 16).

Such high values, however, do not exist in natural waters, except for areas of hydrothermal spring activities and in evaporating basins. The silica level in most rivers, lakes and in the ocean rarely exceeds a few ppm. Thus, for all practical purposes, the hydrosphere is unsaturated with respect to amorphous silica.

V. Enzymic Activities: Carbonate and Phosphate

The two enzyme systems most frequently mentioned in connection with biomineralization are (1) carbonic anhydrase and (2) alkaline phosphatase. Little information, however, has been presented on their specific role in the deposition of minerals. Recent advances in the field of biochemistry may shed new light on this intriguing problem.

(i) Carbonic Anhydrase

Carbonic anhydrase is a zinc-containing enzyme[141, 142] with a molecular weight of approximately 30000[143]. It can occur in the form of multiple isoenzymes which differ in their specific activity[142, 144]. Human carbonic anhydrase B consists of a single peptide chain of 256 amino acids[145, 146]. The space group of its crystalline form is $P\,2_1$ with two molecules per unit cell[147, 148]. Cell dimensions are:

$$a = 42.7\ \text{Å};\ b = 41.7\ \text{Å};\ c = 73.0\ \text{Å};\ \text{and}\ \beta = 104.6\ \text{Å}.$$

A schematic drawing of the molecule is shown in Fig. 17.

The enzyme is quite versatile in that it catalyses the interconversion of CO_2 and HCO_3^- in solution, the hydration of aliphatic aldehydes[149], pyridinecarboxylaldehydes and pyruvic acid[150], and acts as esterases with respect to certain monoesters of carboxylic acids and diesters of carbonic acid[151, 152]. Carbonic anhydrase has been found in a number of animal tissues, plants and a few bacteria[141].

The catalytic mechanism for CO_2 hydration-dehydration by carbonic anhydrase represents the focal issue of the present discussion. We have to consider two aspects: (1) the mode of binding of the CO_2 substrate at the active site, and (2) the physical-chemical state of ligands on the zinc ion.

A direct coordination of CO_2 to the zinc can be excluded. Based on infrared studies it was suggested that the CO_2 molecule is bound to the active site of carbonic

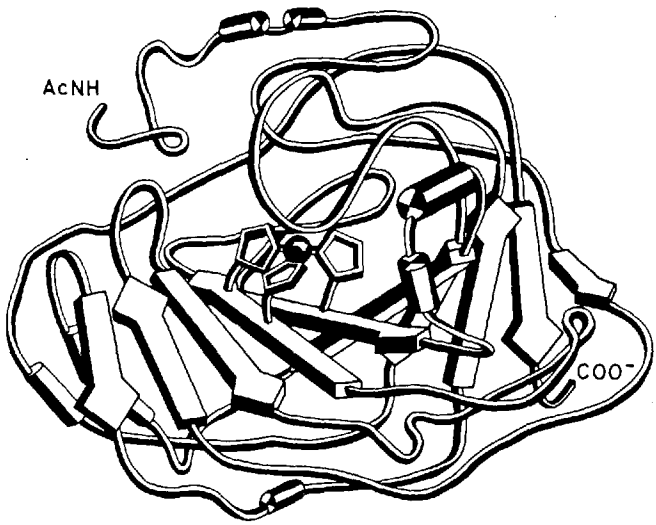


Fig. 17. Idealized drawing of the main chain folding of human erythrocyte carbonic anhydrase C (HCAC). The helices are represented by cylinders and the pleated sheet strands are drawn as arrows in the direction from amino to carboxyl end. The ball supported on three histidyl residues represents the zinc ion (after Liljas *et al.*[147]; Kannan *et al.*[148])

anhydrase in an unstrained fashion and loosely tight to a hydrophobic cavity[153–155]. Since the physical properties of N_2O and CO_2 are perfectly matched, both molecules should thus compete for binding to the active site. This, however, is not the case which implies that in addition to true substrate binding also nonproductive binding of CO_2 is present. It is of note that no general class of competitive inhibitors of the CO_2 hydration activity of carbonic anhydrase has been found to date. All potent inhibitors of enzymic activity become directly coordinated to the zinc atom. This indicates that the CO_2 binding is rather specific, perhaps involving a stereochemical adjustment such as bending of the CO_2 molecule which is known to increase rates of hydration by several orders of magnitude.

There is general agreement that carbonic anhydrase activity is linked to the zinc ion and its ligands. At the active site water is bound to the zinc but the state and involvement of the water in the actual catalysis is a matter of controversy. According to a widely accepted opinion the zinc-bound water is ionized and the activity is a function of pH[156].

The basic form (ZnOH) is active in the hydration reaction, while the acid form ($ZnOH_2^+$) is active in the dehydration reaction. The zinc-bound water participates in both the electron donor and acceptor requirements of the reaction. HCO_3^- is considered the substrate in the dehydration reaction and proton transfer must accompany the breaking of the C–O bond.

This currently accepted viewpoint is in conflict with proton relaxation data[157] which indicate that only the high-pH form of the enzyme has a water ligand and that there is no bound water or OH^- at low pH. The idea has been advanced[157] that only the high-pH form catalyzes both the hydration and dehydration reaction. Further-

more on kinetic grounds there is strong evidence against HCO_3^- as a substrate in the dehydration reaction. Instead, it was proposed that dehydration of HCO_3^- catalyzed by carbonic anhydrase proceeds by the initial formation of a complex between the high-pH form of the enzyme and a neutral H_2CO_3 molecule which displaces the H_2O on the metal. Thus, the reaction that is reversibly catalyzed reads:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3.$$

The idea of H_2CO_3 as substrate for carbonic anhydrase is strongly supported by inhibitor binding studies[158, 159]. Small anion or sulfonamide inhibitors are linked to the zinc ion at high pH. The complex picks up a proton and the inhibitor is bound in a neutral form.

In conclusion, the stereochemistry of the CO_2 at the active site, and the type and state of the ligand at the zinc control the interconversion of CO_2 and HCO_3^- in solution. The rate of catalysis is pH dependent, and the substrate for dehydration reaction is the neutral H_2CO_3 molecule[157].

(ii) Alkaline Phosphatase

Alkaline phosphatase[160–164] is a dimeric zinc metalloenzym composed of two identical subunits. The number of zinc atoms per protein molecule varies in different preparations. However, only two seem to be required for catalytic activity. The molecular weight of the monomer has been reported to be 42.000 so the natural dimer would be twice that value. Alkaline phosphatase is a phosphorylating enzyme and has 760 residues per dimer.

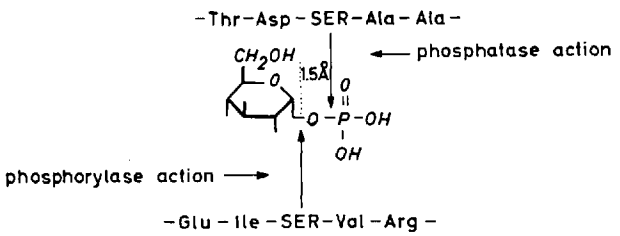


Fig. 18. Release of PO_4 group from glucose-l-phosphate

Zinc can be removed[165] and the resulting apoalkaline phosphatase binds no phosphate with a dissociation constant smaller than 5×10^{-5} M. Upon addition of metal ions to the apoenzyme, a high affinity binding site may be reactivated. Two metals are needed to form one highly specific phosphate binding site.

The action of phosphatase on glucose-1-phosphate substrate is shown in Fig. 18. Phosphatase breaks the P–O bond and releases the PO_4 group. In contrast phosphorylase which operates on the same substrate opens the C–O bond. Apparently this affinity to specific bond sites, which in the present case amounts to a difference of *ca.* 1.5 Å (Fig. 18) between the two bonds "left and right" from the oxygen, is

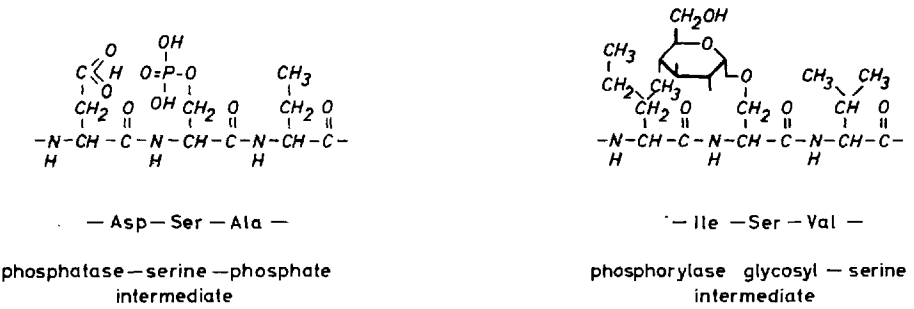


Fig. 19. Intermediates of phosphatase and phosphorylase activities

caused by aspartic acid and isoleucine in the enzymes[166]. The intermediate serine compounds arising from alkaline phosphatase and phosphorylase activity differ although the same substrate, *i.e.* glucose-1-phosphate, is digested by both enzymes (Fig. 19). In the case of phosphatase the phosphate group is split off from serine by water hydrolysis; the phosphate group, however, can also be transfered to an alcohol rather than to a water molecule.

Recent data suggest that all the alkaline phosphatase from mammalian tissues possess pyrophosphatase activity[167, 168] and under special conditions they also have adenosine triphosphatase (ATPase) activity[169]. This contrasts with earlier views that phosphatase and pyrophosphatase activities are due to separate enzymes. The most remarkable finding, however, is the close correlation between calcium ATPase and alkaline phosphatase activities in a series of experiments. The results strongly suggest that we are dealing with the same enzyme[170].

In conclusion, alkaline phosphatase, pyrophosphatase, and a calcium ATPase appear to be the same enzyme. The enzyme has three separate functions: (1) breaking down ATP, (2) hydrolysing inorganic pyrophosphate and possibly other phosphate esters, and (3) opening of C–O–P bonds. In this capacity it has a profound impact on transport and regulation of phosphates and calcium, and is involved in biomineralization processes[171–173].

VI. Calcium Transport and Regulation

Calcium ions are essential in a variety of physiological processes including blood clotting, release of neurotransmitter at the synapse, cell division, cell adhesion, secretion, bioluminescence, membrane permeability, muscle contraction, and biomineralization[35, 174–176]. In most of these systems, the disposition and functions of membrane proteins are a key in transport and regulation of calcium. Thus in order to understand the functionality of calcium one should look at a membrane system where the biochemical interplay of calcium is known in detail.

Research in the field of biomineralization has not yet advanced to a point where we can fully comprehend all the intriguing facets of calcium metabolism. The

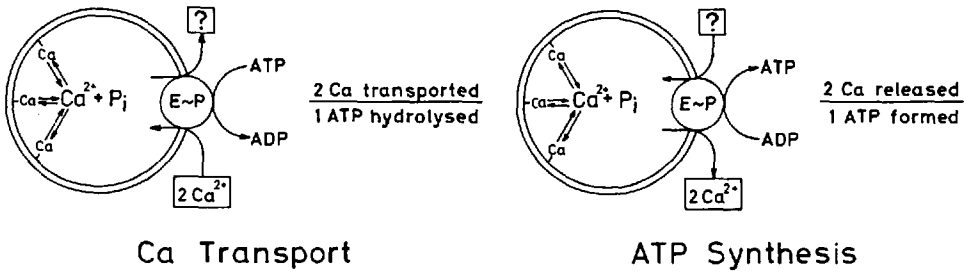


Fig. 20. Relationship between ATP, Ca-translocation, and phosphoprotein in sarcoplasmic reticulum (after Martonosi *et al.*[185])

mechanism of calcium transport and regulation in excitation-contraction coupling in skeletal muscle and sarcoplasmic reticulum, however, is well understood. Since membrane proteins involved in mineralization and muscle activities are: (1) acidic in nature, (2) show strong affinity to calcium ions, and (3) utilise an ATPase as Ca^{2+} transport enzyme, some of the molecular mechanisms at work in one cellular compartment may also operate in the other. In the light of this we will briefly examine the cooperative action of Ca^{2+} ions in sarcoplasmic reticulum membranes and muscle proteins.

(i) Sarcoplasmic Reticulum Membranes

The sarcoplasmic reticulum is a membrane specialised for transport and binding of calcium in muscle tissue[177–180]. Because of this specialisation, the membrane contains only a few proteins[181–183]. Those structurally or functionally involved in the Ca^{2+} transport process are (1) ATPase, (2) a high affinity Ca^{2+} binding acidic protein of 55000 daltons, (3) calsequestrin of 55000 daltons, (4) a set of three acidic proteins with molecular weights ranging between 20000 and 32000, and (5) a low molecular weight proteolipid. The first three account for more than 95% of the total vesicular protein and the ratio ATPase: calsequestrin: high affinity Ca^{2+} binding protein is about 7 : 2 : 1[179].

The Ca^{2+} transport of sarcoplasmic reticulum is energized by hydrolysis of ATP, acetylphosphate, or carbamylphosphat through a transport ATPase which is tightly bound to the microsomal membrane[184, 185]. The relationship between ATP, Ca^{2+} translocation, and a phosphorprotein intermediate is depicted in Fig. 20. Transport of Ca^{2+} into sarcoplasmic reticulum vesicles is accompanied by the hydrolysis of ATP by means of a phosphorprotein intermediate and by ADP formation. The phosphorylenzyme is most likely an acylphosphate. For one mole of ATP two calcium atoms are transferred across the membrane where they are bound to membrane linked cation-binding sites. Reversal of this process leads to hydrolysis of phosphor protein, liberation of orthophosphate, and release of two calciums; the carrier will return to the cytoplasmic surface. In summary, the ATPase protein represents a complete transport system containing both the site of ATP hydrolysis and the Ca^{2+} carrier within a single molecule.

During muscle relaxation and contraction, calcium ions are constantly on the move from one site to another. A major site of Ca^{2+} sequestration in the interior of the sarcoplasmic reticulum is calsequestrin[179]. Others assume that calsequestrin is located at the exterior face of the sarcoplasmic reticulum membrane and the role of calcium binding could possibly be exercised by the high affinity Ca^{2+} binding acidic protein of 55000 daltons. However, the weight of evidence suggest that calsequestrin is not located at the exterior, and that the functions of the two major Ca-binding proteins inside the vesicle are intertwined[179].

High affinity Ca^{2+} binding sites amounting to about 10 nmoles per mg of protein are exposed on the exterior of sarcoplasmic reticulum membranes. They can solely be accounted for by the ATPase which binds two moles of Ca^{2+} per mole and constitutes between 5 and 7 nmoles per mg of intact sarcoplasmic reticulum protein. Inside the vesicles the binding capacities for ATP-involved calcium can be as high as 100 nmoles per mg protein.

The carrier-mediated active transport system of calcium is responsible for the relaxation of muscle. However, the rate of efflux from sarcoplasmic reticulum membranes during reversal of the transport process is 10^2 to 10^4 orders too low to account for the massive calcium release from sarcoplasmic reticulum in stimulated muscle. Instead, passive diffusion of calcium across the sarcoplasmic reticulum membrane will proceed during excitation of muscle[178, 179, 186]. The rate of calcium release observed during excitation is 1.000–3.000 μ moles/mg protein/min which is an increase of about 10^4 to 10^5 over the resting state.

Phospholipids which constitute close to 40 percent of the mass of the sarcoplasmic reticulum membranes dominate the permeability characteristics through formation of liposomes which are capable of scavenging calcium ions and carry them across the membrane[178]. The permeability properties of liposomes depend on their phospholipid content. Rate of calcium release markedly increases with increasing temperatures[187, 188] and in the presence of local anesthetics and microsomal proteins[178]. When contraction is initiated by a nerve impulse, the impulse travels through the T-system (transverse tubules). Depolarisation of the system causes release of calcium from the sarcoplasmic reticulum[189]; depolarisation may cause a potential change across the sarcoplasmic membrane which in turn induces the release of calcium. For recent reviews on sarcoplasmic reticulum membranes, see Ref.[177–179].

(ii) Muscle Proteins

Four major proteins – myosin, actin, troponin and tropomyosin – are contained in muscle fibers of vertebrates[190, 191, 191a].

Contraction-relaxation processes in muscle proceed by a sliding filament mechanism, whereby actin and myosin filament move relative to one another. The reaction is energized by ATP and regulated by the level of calcium ions. In vertebrate skeletal muscle contraction is controlled by the interaction of calcium ions with the specific protein troponin which is attached to the actin filaments, whereas among many invertebrates troponin is lacking and myosin and not actin is controlled[192]. However, in a few invertebrates both types of filaments are involved in regulation.

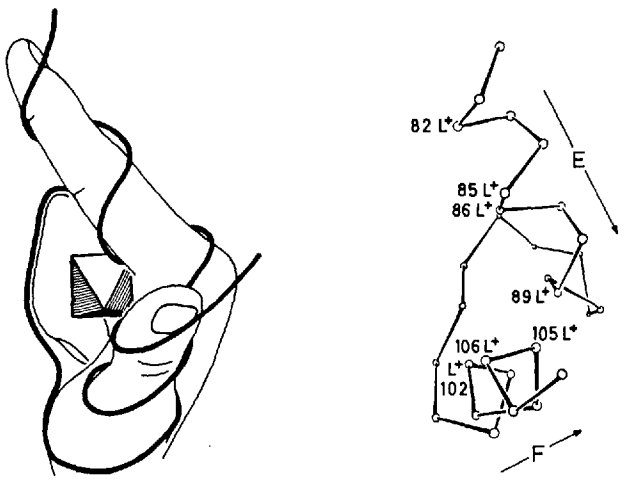


Fig. 21. The symbolic EF hand on the left represents helix E (forefinger), the calcium binding loop (middle finger enclosing an $Ca^{2+}O_6$ octahedron), and helix F (thumb). The α-carbon skeletal models of the carp MCBP EF hand (right) (after Tufty and Kretsinger[203])

The message of calcium is conveyed from the troponin to which it binds, via tropomyosin to the actin filament[193–198]. As soon as the nerve impulse ceases, the calcium becomes quickly removed and returned to the storage sites situated in the membranes of the sarcoplasmic reticulum. When Ca^{2+} concentration in the sarcoplasm reaches 1×10^{-7} molar, the fiber is relaxed.

Protein sequence and structure of troponin (TN–C) and carp muscle calcium binding parvalbumin (MCBP) are known[199–201]. MCBP has two calcium binding regions each consisting of an α-helix, a loop about the calcium ion and another α-helix, as so-called EF hand (Fig. 21)[202, 203].

The calcium binding component of troponin from rabbit muscle contains four homologous EF hands. It was proposed that the structure of TN–C is rhombic and consists of two pairs of EF hands in approximate point group symmetry (space group: 222).

Four calcium ions are bound to TN–C. The amino acid sequence in the four calcium binding regions of TN–C and the two regions of MCBP (CD and EF hand) are depicted in Fig. 22. Calcium ions are present in octahedral coordination – $Ca^{2+}O_6$ – and different oxygen ligands are incorporated in the calcium ion coordination complex. In the case of TN–C, acidic amino acids provide 17 of the 24 oxygens needed for the four calcium octahedra that can become part of one TN–C molecule. This emphasises the significance of carboxyl groups for coordination purposes. Judged by the invariability of amino acids at the X (ASP) and –Z (GLU) vertices of the Ca^{2+} coordination polyhedra in all six calcium-binding regions shown in Fig. 22, it appears that the presence of these two amino acids at these sites is crucial for the structural incorporation of calcium ions and may also be essential for the 3-D alignment of the two helices. The invariability of amino acids next to the amino acid at the – Y vertix-(GLY-[PHE, LYS, ASP, THR, TYR, ARG]-ISO) – and

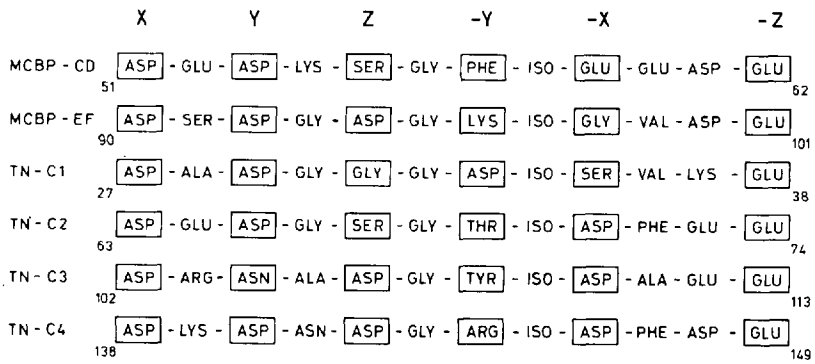


Fig. 22. Comparison of EF hand amino acid sequences in carp muscle parvalbumin (MCBP) and calcium binding component of troponin (TN-C) (after Collins *et al.*[199, 201]; Collins[200]; Tufty and Kretsinger[203])

the seemingly arbitrary selection of amino acids that occupy this coordination site imply that the oxygen ligand is provided by the peptide group and that strong steric control is excercised by the glycine and isoleucine residues.

It was suggested that many proteins involved in calcium transport and regulation are homologous and would contain one or several EF hands[205]. On the basis of computer alignment scores this suggestion appears to be valid for some calcium binding proteins. Other proteins not necessarily connected to calcium regulation score high on the alignment list indicating the existence of a pair of α-helices. The presence of EF hands may simply be a thermodynamically preferable conformation. It is the presence of carboxyl groups at specific coordination sites which is the most probable control mechanism for Ca^{2+} ion fixation.

In conclusion, troponin binds Ca^{2+} in six-fold coordination and all oxygen ligands are provided from amino acids. No water is needed for the coordination complex. Aspartic acid and glutamic acid occupy key positions in the four calcium binding regions of the molecule. Transfer of calcium to tropomyosin and the actin filament during contraction is achieved by coordination changes.

In molluscan muscles and in some primitive invertebrates, contraction is mediated by the myosin and not by the thin filaments[192]. The calcium dependence of the actin-activated ATPase activity of molluscan myosin requires the presence of a specific light chain (EDTA-light chain). This light chain has a molecular weight of 18000 daltons. Removal of EDTA-light chains from myosin preparations will lower the amount of calcium by about 40%. The amino acid composition of the light chain is characterized by the high abundance of aspartic acid and glutamic acid which account for about one third of the residues.

The exact manner in which calcium and the EDTA-light chain regulate the myosin ATPase is unclear. The fact that isolated EDTA-light chain does not bind calcium suggests that all of the calcium is bound to the heavy chains and that the light chains only introduce conformational changes[192]; specific binding sites appear to be involved[204].

A remarkable experiment[205] has been performed on the function of myosin light chains in calcium regulation. It shows that rabbit DTNB (5,5-dithiobis-2-nitro-

benzoic acid) light chain can be bound to densensitised scallop myosin and restore calcium sensitivity. DTNB- and EDTA-light chains seem to be interchangable. The amino acid sequence of light chains of rabbit skeletal muscle-myosin has been determined[206] and aspartic and glutamic acids show the same concentration levels as in scallop EDTA-light chain.

In summary, interaction between actin and myosin in vertebrate and invertebrate muscle systems requires a critical level of calcium. It is tentatively suggested that interaction between myosin and actin is prevented by blocking sites on actin in the case of vertebrate muscles, whereas in the case of molluscan muscles it is the sites on myosin which are blocked in the absence of calcium. The acidic amino acids seem to be of utmost importance in the regulation process.

VII. Carbonate Deposition

(i) Organic Matrix Concept

All skeletal material consists of two phases: an organic and an inorganic one. The ultrastructural relationships are commonly explained in terms of the organic matrix concept, which implies that organic matter serves as a template in the nucleation and oriented growth of biominerals. Little attention has been given the possibility of organic molecules acting as inhibitors in the sense of confining mineral growth to a limited space.

One of the major difficulties in the interpretation of ultrastructural patterns is the realisation that one is dealing with relic structures that are "frozen" in mineral matter. There is no way to be sure that the shape and composition of the mineral matrix and even the mineral itself has remained the same. They may be partly or entirely different from the state both had at the time of formation. Some intermediary products involved in the regulation no longer exist and we are only left with the final products.

The amount of organic matter in skeletal material can be as low as 0.01 percent in some mollusc shells, and as high as 20 to 30 percent in vertebrate bones or teeth; in a few isolated instances concentrations may go up to 90 percent. The origin, nature and function of mineralized tissues in calcification is only tentatively known. It is the objective of the following discussion to coordinate in a meaningful way the often conflicting results found in the literature on mineral deposition processes.

(ii) Structure of Organic Matrix

Aragonite and calcite are the two calcium carbonate species most frequently encountered in biological and geological systems[7]. Their major domain are the lower plants and invertebrates and they rarely occur in higher organisms. One of the few exceptions are otoliths, which are small aragonitic stones weighing a few milligrams to grams deposited in the inner ear of fishes. Another example are egg shells of birds and reptiles.

In most instances, calcium carbonates are associated with a proteinaceous matrix composed of proteins and glycoproteins[109–111, 207–221]. On a gross compositional basis there is little difference in the amino acid content of carbonates formed biologically and those deposited chemically (Table 2)[220]. Both groups are characterized by a predominance of aspartic acid. Recently, attention has been drawn to the fact that some ancestoral forms – corals and algae – contain high amounts of sugars and uronic acid in their skeletal material[111, 222, 223]. Thus the possibility exists that in addition to proteins and glycoproteins also carbohydrates may function as a template in carbonate deposition.

Table 2. Amino acid composition of various carbonates, in residues/1000[109, 110, 220]

Amino acid	*Hydroides* (Serpulid) (Worm tubes)	*Porites* (Scleractinian) (coral)	*Eunicea* (Alcyonarian) (coral)	Oolites (Bahamas)	Aragonite Needle mud (Bahamas)
Aspartic acid	213	344	745	302	313
Threonine	97	21	13	51	50
Serine	42	29	13	46	54
Glutamic acid	120	100	37	109	121
Proline	62	35	11	38	35
Glycine	102	114	79	139	141
Alanine	90	67	60	93	79
Cystine (half)	4	2	1	2	2
Valine	72	56	14	46	43
Methionine	8	11	1	24	20
Isoleucine	41	46	5	28	24
Leucine	49	69	6	52	42
Tyrosine	18	10	1	7	7
Phenylalanine	36	33	3	38	26
Ornithine	4	11	1	2	3
Lysine	18	27	5	14	22
Histidine	3	12	1	2	2
Arginine	21	13	4	7	16
Total (μmoles/g $CaCO_3$)	8.4	2.9	17.2	3.8	21.8

Heating experiments on *Nautilus* shell material (see page 10) indicates a retardation in the rates of thermal decomposition of mineral and organic phase. This has been attributed to the development of a topochemical boundary at the contact protein-mineral. X-ray diffraction analysis gives no evidence for structural alteration of the original aragonite during the thermal treatment up to 200 °C.

The organic matrix follows the same pattern. By means of electron diffraction analysis it can be shown that the protein matrix is and remains crystalline and that the structural order of the diffraction pattern is that of a microcrystalline film of gold (Fig. 23)[224]. It is remarkable that the matrix proteins in other species also produce a single crystal spot pattern. This implies that isolated pieces of organic matrix which are in the order of a few microns thick represent single crystals. Whatever the term "single crystal" may mean to the various researchers[43], in the context

(a) (b)

Fig. 23. (a) Electronmicrograph of organic matrix of *Mercenaria mercenaria*. (b) Electron diffraction pattern of organic matrix proteins shown in (a)

of the present work it is used to demonstrate that the protein matrix in shell structures represents a well-defined crystalline network.

(iii) Chemistry of Organic Matrix

In Table 3, the geometric means of amino acids in mineralized tissues of a series of representative classes of organisms are sumarized[217, 218, 225]. In spite of the diversity in the amino acid composition of the shell organic matter, all matrices have one property in common, that is, they contain functions that operate as an effective calcifying agent.

On a relative basis, *i.e.* residues per 1000, there is virtually no one species like the other. In contrast, different shell samples from the same species and obtained from the same natural habitat yield identical amino acid patterns. It is of interest that (1) the structure of carbonates (aragonite-calcite-vaterite), (2) the content in trace elements, and (3) the stable isotope distribution are markedly effected by fluctuations in salinity, water temperature, Eh/pH conditions, and some anthropogenic factors. The same environmental parameters determine to a certain degree the chemical composition of the shell organic matrix. This feature suggests a cause-effect relationship between mineralogy and organic chemistry of a shell. In the final analysis, however, it is simply a reflection of the environmentally-controlled dynamics of the cell.

To explain the species-specific amino acid pattern one can assume that each organism secretes one particular calcifying protein which to a limited extent is controlled by the environment. Alternately, a series of different proteins can yield the observed pattern, however, we have to postulate that each one is secreted at a constant rate. Changes in rate of secretion, perhaps environmentally induced, will effect the ratio of the individual proteins and thus account for slight variation in the amino acid distribution in the hydrolyzed matrix.

Since at the species level virtually no difference in distribution of amino acids exists, unless specimens are taken from diverse environments, it was of interest to

Table 3. Amino acid composition of organic matrix in shell carbonates (geometric means in Res./1000; numbers in parentheses are 1σ ranges)[218)]

	Amphi-neura (1)	Cephal-opoda (4)	Gastro-poda (40)	Pelecypoda (51)	Brachio-poda (1)	Bryozoa (3)	Echinoidea (4)	Portunid-crabs (6)	Pisces (Otoliths)	Avian Shell (Goon)
OH-proline	–	–	.01 (0–0.1)	–	–	0.1 (0–7)	1 (0–36)		30	–
Aspartic acid	128	98 (75–128)	114 (69–185)	136 (77–240)	97	117 (100–136)	88 (78–98)	81 (73–92)	162	100
Threonine	60	34 (18–64)	46 (28–76)	28 (18–44)	39	59 (53–66)	48 (42–55)	57 (53–61)	57	66
Serine	68	95 (62–146)	85 (58–124)	66 (43–102)	70	77 (60–98)	81 (77–86)	91 (80–105)	44	101
Glutamic acid	98	65 (42–128)	87 (52–145)	50 (35–72)	77	102 (93–112)	109 (106–112)	94	170	128
Proline	93	55 (15–205)	61 (36–102)	52 (28–98)	36	50 (36–67)	78 (66–92)	103 (89–119)	50	72
Glycine	131	153 (89–262)	153 (99–238)	271 (185–398)	301	148 (127–172)	171 (121–241)	113 (101–125)	125	130
Alanine	88	126 (74–216)	89 (52–153)	66 (43–101)	76	92 (89–95)	85 (79–91)	115 (102–130)	96	86
Cystine (half)	14	7 (0–65)	9 (4–23)	14 (6–33)	8	13 (4–39)	1 (0.5–2)	3 (1–9)	17	36
Valine	45	36 (20–64)	48 (34–69)	33 (22–49)	74	48 (38–60)	38 (31–45)	65 (59–72)	70	53
Methionine	12	8 (5–13)	14 (6–31)	18 (8–39)	13	16 (14–18)	21 (18–25)	7 (3–11)	1	1

Table 3 (continued)

	Amphi-neura (1)	Cepha-lopoda (4)	Gastro-poda (40)	Pelecypoda (51)	Brachio-poda (1)	Bryozoa (3)	Echinoidea (4)	Portunid-crabs (6)	Pisces (Otoliths)	Avian Shell (Goon)
Isoleucine	30	21 (17–26)	31 (20–46)	20 (13–31)	37	31 (25–39)	26 (21–32)	28 (25–31)	30	29
Leucine	52	44 (29–67)	71 (51–100)	34 (26–44)	47	59 (46–76)	58 (47–70)	49 (44–54)	73	63
Tyrosine	31	33 (15–75)	11 (5–24)	11 (2–61)	4	23 (21–25)	20 (16–25)	25 (20–30)	1	24
Phenylalanine	37	30 (20–45)	24 (14–43)	30 (19–48)	19	28 (24–33)	25 (19–32)	30 (24–37)	12	38
OH-Lysine	–	–	.03 (0–0.2)	.03 (0–0.6)	0.1	0.4 (0–8)	0.6 (0–11)	3 (0–7)	–	–
Lysine	41	20 (5–83)	26 (17–41)	22 (13–38)	31	49 (40–61)	45 (35–57)	52 (28–97)	20	17
Histidine	11	5 (0–35)	3 (1–16)	4 (1–14)	0.3	17 (10–30)	16 (13–20)	26 (17–39)	7	19
Arginine	61	23 (10–56)	22 (9–52)	24 (6–96)	70	50 (41–60)	69 (60–80)	35 (18–68)	14	36
Protein/ hexosamines[1])	0.98	1.37	108.30	143.97	110.97	21.93	33.36	0.40	96	54

[1]) From arithmetic means.

Table 4. Amino acid composition of shell proteins in *Haliotis* (residues per 1000)

	sorenseni	*fulgens*	*corrugata*	*assimilis*	*walallensis*	*kamtschatkana*	*cracherodii*	*rufescens*
OH-Proline	5	2	3	2	1	5	3	3
Aspartic acid	197	132	150	189	180	203	180	203
Threonine	12	21	24	19	19	21	17	18
Serine	119	84	94	118	114	119	127	120
Glutamic acid	49	57	55	37	56	48	49	52
Proline	48	152	96	35	46	39	36	20
Glycine	220	166	172	216	186	216	207	207
Alanine	144	80	102	149	141	159	164	155
Cystine (half)	4	5	4	6	2	6	2	1
Valine	27	39	41	32	62	26	30	28
Methionine	2	3	4	3	10	2	4	1
Isoleucine	10	8	10	10	13	10	12	11
Leucine	27	17	24	29	33	28	31	29
Tyrosine	21	129	90	14	20	14	14	15
Phenylalanine	33	18	23	42	34	63	35	42
OH-Lysine	1	1	1	1	1	1	2	1
Lysine	12	32	26	27	18	10	21	14
Histidine	3	1	12	3	9	1	1	3
Arginine	66	62	69	68	55	29	65	77
Total proteins[1])	182	392	199	112	118	73	109	142
Glucosamine[1])	3.85	1.83	3.35	3.50	2.69	1.24	0.41	3.75
Galactosamine[1])	0.15	0.37	0.60	0.27	0.23	0.08	0.05	0.16
Total hexosamines[1])	4.00	2.20	3.95	3.77	2.87	1.32	0.46	3.91
Protein/hexosamines	46	178	50	30	41	55	237	36

[1]) μmoles/g $CaCO_3$.

Table 5. Amino acid composition of periostracum in *Haliotis* (residues per 1000)

	sorenseni	*fulgens*	*corrugata*	*assimilis*	*walallensis*	*kamtschatkana*	*cracherodii*	*rufescens*
OH-Proline	–	–	–	–	–	–	–	–
Aspartic acid	357	244	360	306	209	190	296	242
Threonine	34	34	13	31	31	31	30	30
Serine	56	53	37	65	50	44	52	37
Glutamic acid	67	96	46	77	93	97	97	103
Proline	16	48	18	22	69	79	40	51
Glycine	288	242	309	261	233	228	237	263
Alanine	25	37	29	48	48	38	30	33
Cystine (half)	14	7	31	2	6	7	16	7
Valine	23	19	27	38	31	30	33	27
Methionine	1	1	2	4	1	1	2	3
Isoleucine	14	22	21	23	28	24	26	22
Leucine	22	28	30	34	26	20	28	14
Tyrosine	7	57	11	12	63	61	40	55
Phenylalanine	10	21	12	32	38	50	32	24
OH-Lysine	1	1	9	1	2	1	1	2
Lysine	28	36	29	32	26	32	30	32
Histidine	7	6	12	4	9	4	4	10
Arginine	30	48	4	8	37	63	6	45
Protein/hexosamines	86	355	32	85	171	378	38	105

examine species variation within one genus and to compare relationships among amino acids in shell matrices and unmineralized periostraca. Data from an ancestoral gastropod, *Haliotis*, are discussed below, which were taken from the sea off California[226].

Shell matrix proteins in *H. fulgens* and *H. corrugata* are distinguished from the six other species investigated by their lower concentrations of aspartic acid, glycine, alanine, serine and phenylalanine, and higher concentrations of proline and tyrosine (Table 4). The levels of the remaining amino acids are about the same for all specimens. A brief glance at Table 4 reveals distinct correlations between (1) glycine and aspartic acid, (2) tyrosine and proline, and (3) serine and alanine. Actually, most of the observed variations among the eight species are due to fluctuations of these three factors.

The high serine, alanine, glycine, proline and tyrosine content suggests that one of the protein fractions in the shell matrices is related to a silk-type protein. For example, enzymatic degradation of silk releases peptides such as Gly-Ser-Tyr-Pro and tyrosylproline. The relationship between serine and alanine suggests a fibrinogen. Another peptide fraction enriched in glycine and aspartic acid and comprising 50 to 60 per cent of the total organic matrix, suggests the existence of an acidic protein.

In the shell proteins, the Gly-Asp and Ser-Ala fractions are almost linearily related; observed differences in amino acids among the eight *Haliotis* samples are caused by variations in fraction 3, *i.e.* Tyr-Pro.

In the periostraca (Table 5), the Ser-Ala fraction is constant; the observed increase in Gly-Asp is partially compensated by a relative decrease in Tyr-Pro content.

Fig. 24. Electronmicrograph of crystals formed from 2 molar $CaCl_2$ extracts of *Mercenaria mercenaria* following removal of salt. Negatively stained with 1% uranyl acetate (x 50000). A characteristic staining pattern is developed with well-defined periodicities. This material represents the MM. The crystals and staining pattern closely resemble tropomyosin-troponin paracrystals[193]

Therefore, it appears that the amino acid spectrum in shell matrix proteins and periostraca of *Haliotis* is largely a result of the mixing of three different peptide fractions, each one characterized by two amino acids. The six amino acids involved in these three fractions account for about 70 to 80 per cent of the total. The principal difference between shell matrix proteins und periostraca proteins is the high abundance of alanine and serine in the calcified tissue which suggests that the fraction containing these two amino acids is critical in biomineralization processes.

Several attempts have been made to bring individual proteins and peptides in solution[227] using enzymatic and non-enzymatic degradation techniques. Tryptic digest does not result in the dissolution of shell matrix proteins, but a treatment with urea, hydroxylamine and formic acid partially degrades the tissues. Hydroxylamine treatment results in the release of units of molecular weight of 20000 to 80000. Of the solvents tested, most effective were 1 to 3 molar $CaCl_2$ or concentrated glucuronic acid solution. Both yielded the same peptide fraction. A subsequent treatment of the residue with strong hydrogen bond breaker such as urea, brought an additional peptide increment into solution.

Upon removal of the calcium ions by dialysis or extraction of the glucuronic acid, a white precipitate forms which is highly enriched in aspartic acid (~50%), glycine and serine. This set of amino acids provides sufficient oxygen ligands (COOH, OH, peptide link) for the coordination of Ca^{2+}. Strong steric control is probably exercised by glycine residues. An electron micrograph of the precipitate is shown in Fig. 24 and crystalline aggregates are indicated.

Water soluble proteins are present in shells in the amount of 0.1 to 2% of the total organic matter. EDTA treatment will release additional increments and in *Mercenaria mercenaria* shells, 15% of the total is EDTA extractable[228]. The material represents a highly sulfated glycoprotein with a molecular weight in the order of 160000.

(iv) Template Model

Chemical studies on *Nautilus pompilius* (a cephalopod) and *Mercenaria mercenaria* (a clam) indicate that matrix proteins are composed of two structural units: (1) a polypeptide with a strong affinity to Ca^{2+} ions which is soluble in strong $CaCl_2$ solutions, and (2) a high molecular weight protein insoluble in $CaCl_2$ with no affinity to calcium and which is arranged in the form of sheets and layers.

Based on these and previous data, the following mineralization mechanism is proposed. One protein fraction which we will term the "Carrier Protein" (CP), is secreted by the organism and will polymerize in the form of sheets. It serves as a carrier and binding agent for an acidic polypeptide fraction which has a strong affinity to calcium ions. This polypeptide will be designated as the "Mineralization Matrix" (MM).

The attachment of the MM to the CP will activate the mineralizing substrate and calcium carbonate crystals are laid down in epitaxial order. Crystal growth terminates upon secretion of a newly formed MM on the growing crystal surface whereby a kind of organic blanket is formed. Subsequently, CP becomes attached to MM. A sand-

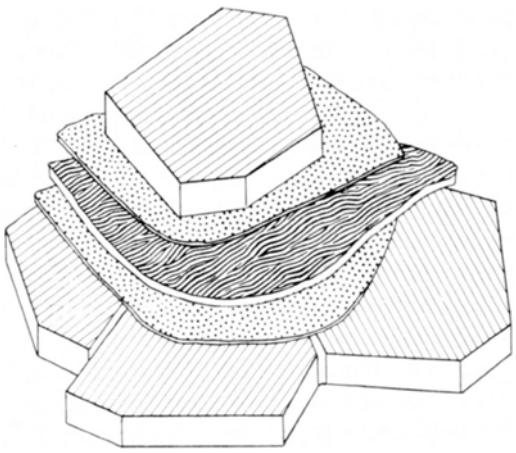


Fig. 25. Schematic representation of structural relationship between layers of MM (dotted), CP (banded) and $CaCO_3$ (block-shaped)

wich structure is thus generated with the CP embedded between two sheets of the MM (Fig. 25). In this way, the organic substrate is activated and calcification may start again. Thousands of such layers can be generated in the life time of an organism. Whereas MM forms a cover only a few Å thick, CP may have a thickness ranging from 30 Å to several hundreds of Å depending on the species. For comparison, the size of the individual $CaCO_3$ crystals is commonly on the order of a few microns.

In *Nautilus*, the CP has a ratio of GLY : ALA : SER = 3 : 2 : 1. This ratio is identical to that described for the β-configuration of silk fibroin, where serine supplants alanine in the layer structure in concentrations up to 15 mole %[229]. In silk, polypeptide chains are arranged in a zig-zag configuration and so-called pleated sheets are generated (Fig. 26).

The structure is maintained by hydrogen bonding between adjacent chains[229]. An identical structure is envisioned for the CP. It is of note that the level of serine in shell matrix proteins is commonly arround 8–10 mole % and may reach concentrations as high as 20 mole %[217, 218]. The main function of serine lies in its ability to form hydrogen bonds which increase the degree of cross-linkage among protein chains and raise their hydrophobic nature[166, 230]. The oxygen of the hydroxyl group can form two hydrogen bridges by supplying one H-atom and attracting another one. This process increases the dispersion bonding forces between residues by bringing these groups into closer contact. In this way the proteins become remarkably resistant to chemical influences. This may explain why organisms adopt silk-type proteins as an inert substrate in mineralization processes.

The MM has a different structural order. The most probable arrangement involves a spiraled peptide chain, which gives rise to a hexagonal network. A structural analog is found in the B chain of insulin which is composed of 30 residues (Fig. 27). Its carbonyl oxygens are located at the corners of nine continuous regular hexagons which are arranged in a honeycomb pattern[231]. All carbonyl oxygens are positioned

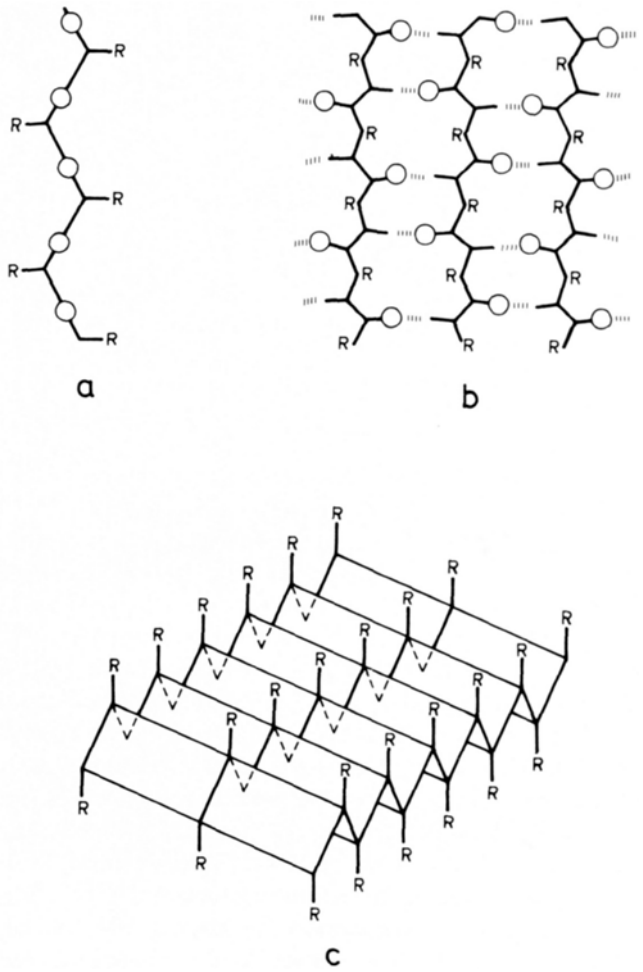


Fig. 26. Schematic representation of β-pleated sheets; (a) top view, (b) edge view, and (c) three parallel chains in β-structure. Hydrogen bonds (IIIII) are indicated in (b)

in a single plane thus producing a hydrophilic surface. The side chains are located on the opposite side and they generate a hydrophobic surface.

The attachment of the MM to the CP may possibly involve the incorporation of water which would link the two fractions via hydrogen bonds. Sterically, the water would fit perfectly into such a network and a greater stabilization would be achieved. The presence of water in shell structures either within the mineral or associated with the intracrystalline matrix has been reported[232]. On the other hand, the high amount of serine in the CP could provide the necessary hydrogen bridges.

The microarchitecture of shell carbonates can be reduced to a few structural elements in spite of the enormous morphological details developed by the organisms.

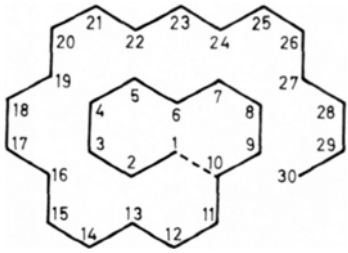


Fig. 27. Space-filling model of the B chain of insulin. Numbers indicate position of carboxyl oxygens (after Warner[231]). The MM in nacreous layer of molluscs is assumed to be built according to the same principles

A particularly well-studied structure is that of the nacreous layer (= Mother-of-Pearl) of molluscs, which will be used below to illustrate our concepts[17, 34, 46, 233–241].

Nacre consist of a sucession of mineral laminae which are intercalated with organic matter commonly termed "conchiolin". In Fig. 28 terraces, imbricate growth surface pattern, and terminating screw dislocations in a pelecypod shell can be recognized. The incipient euhedral crystals (aragonite) are aligned with their orthorhombic *b* axes parallel to the horizontal growth direction of the shell. A hypothetical cross-section showing the structural association of mineral and its coexisting organic phases is attempted in Fig. 29; it is a two-dimensional projection. The MM not only serves as nucleating agent but restricts mineral growth along *c* simply by providing a limiting cover. Growth can only continue along b, *i.e.* by enlargement of the crystals laterally. Screw dislocations may represent the termination or start of a polypeptide chain[242, 243]. Also the systematic spirals composed of aragonite crystals which are spread out across the nucleating surface[237, 243, 245] may well be linked to the conformation and spiraling growth pattern of MM. The key to the whole process is the function of MM as a starting and terminating device in mineral growth.

For continuation of mineral growth in the vertical direction, a CP has to be secreted and for reasons of conformation a phase shift is introduced. This may account for the brick-wall pattern in pelecypod or the zig-zag-pattern in stacks of gastropod nacre. Structures of this type resemble clay minerals, which are also capable of incorporating structural water between metal ion polyhedra.

In Fig. 30, a three-dimensional model is presented in which only the organic phases are shown. Hexagonal plates of MM alternate with pleated sheets of CP. The hydrophobic sides of MM are facing each other and encase the mineral phase. The relationship between hydrophobic bonding and accessible surface area in proteins, and the effect of polar and non-polar side groups on free energy values has recently been discussed[246]. For informations on hydrophobicity in protein systems see Refs.[247–252].

The proposed calcification model offers a compromise between the two conflicting schools of thought, *i.e.* the epitaxis and compartment schools[43]. Both viewpoints are consistent with the mechanisms here proposed.

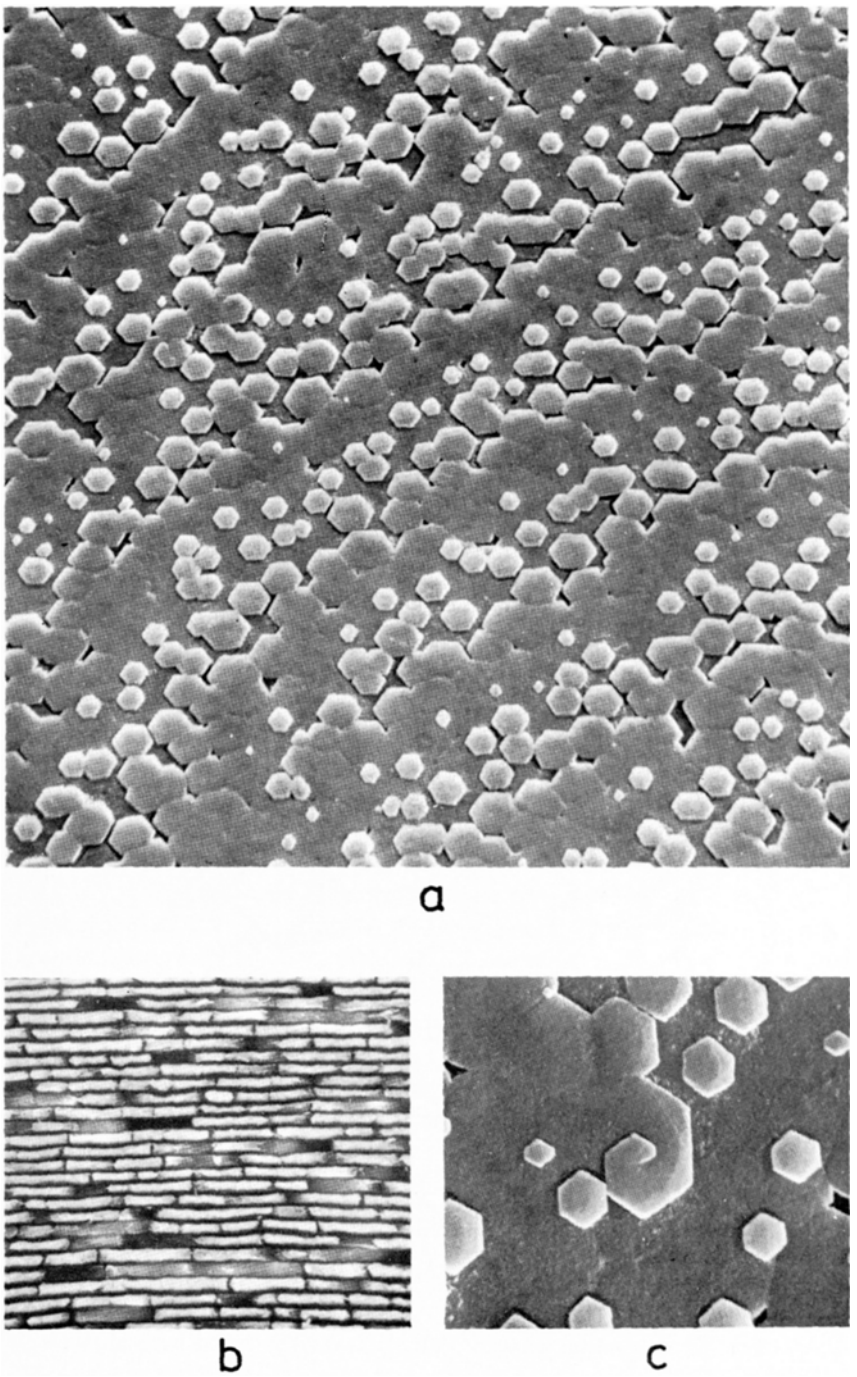


Fig. 28. (a) Electronmicrographs (a) taken in ventral region of pelecypod nacre showing steps formed by overlapp of mineral laminae on growth surface; (b) vertical cross section of pelecypod nacre exhibiting brick-wall pattern; (c) terminating screw dislocation (after Wise and deVilliers[243])

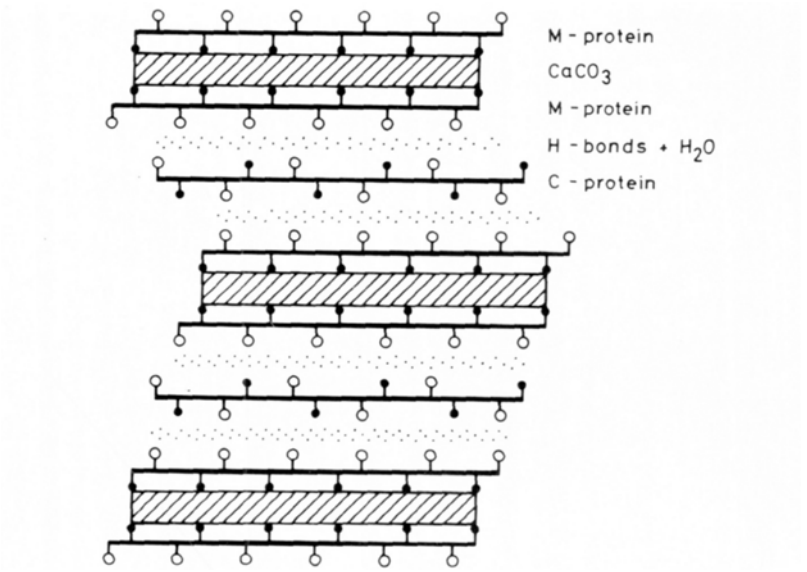


Fig. 29. Schematic representation of vertical cross section of nacre. R-groups are indicated as black dots and oxygens as open circle. M = mineralizing matrix and C = carrier protein

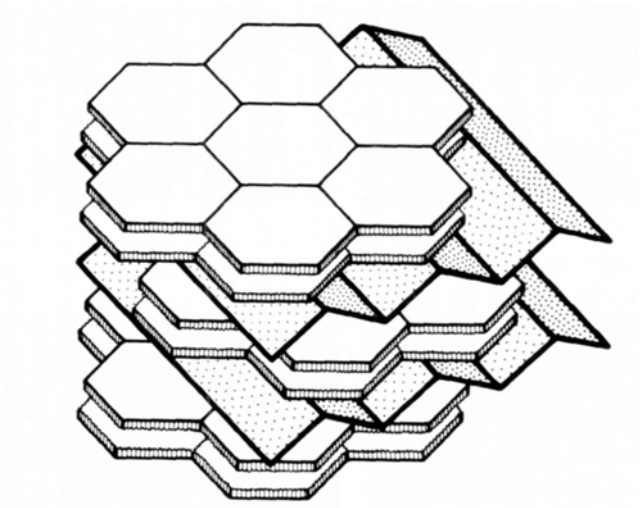


Fig. 30. Schematic representation of vertical cross section of nacre showing alternating layers of mineralizing matrix (hexagons) and carrier protein (pleated sheet conformation). The $CaCO_3$ has been omitted for graphical reasons

(v) Enzymic Control

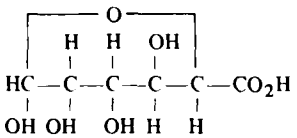
So far, we have presented certain concepts on carbonate deposition using a template mechanism which is based on solid state principles. One should, however, bear in

mind, that there are many unopen questions still to be answered most of which are related to transport and regulation of calcium and carbonate prior to and at the onset of calcification. The organic and inorganic components of the shell structures are only the endmembers of a long physiological production line which is hidden inside the active cell.

Two observations may help to elucidate these points: (1) the sensivity of $CaCO_3$ nucleation and growth to a wide variety of ions and molecules present in the environment, and (2) the ease of solubilisation of the MM by $CaCl_2$ and glucuronic acid.

There is no energy problem in $CaCO_3$ deposition since the overall crystallisation will liberate energy on the order of 2–4 kcal/mole. To allow $CaCO_3$ deposition – which must proceed rather quickly – the surface must be kept clean from interfering components. This may be done by chemical species such as sugar derivatives and their action may be compared to that of jet gases on the crystallisation of ice at high altitudes. Potential inhibitors in $CaCO_3$ formation are a variety of phosphate molecules[253] and magnesium ions which have to be removed before seed formation can be initiated.

It was shown (p. 37) that glucuronic acid:



can solubilize MM.

This may suggest that cellular systems involved in calcification utilize glucuronic acid or a similarly acting carbohydrate as a carrier for a polypeptide which later becomes the MM. The following scenario is tentatively proposed.

Glucuronic acid forms a soluble and inactive complex with the mineralizing polypeptide. This complex is transported to the nucleation site. Here, the carboxyl group might become transformed into an acetyl group thereby rendering the molecule chemically inactive. Alternately, an enzyme – a protease – may open the polysaccharide peptide complex. It is of note that epiphyseal cartilage loses polysaccharide prior to the deposition of mineral through the action of a protease. Removal of glucuronic acid causes polymerization of the MM and its subsequent attachment to the CP. The freely exposed hydrophobic site will immediately function in the fixation of calcium ions and the formation of $Ca^{2+}O_6$ and $Ca^{2+}O_9$ coordination polyhedra. There is an indication that also amino sugars are linked to the active calcium transport. In the light of this, glucuronic acid together with glucosamine may not only serve as building blocks of cell walls (*e.g.* hyaluronic acid) but also act as a carrier in calcification.

Several lines of evidence indicate that carbonic anhydrase is related to calcification in two possible ways (1) as a simple catalyst of CO_2 hydration, and (2) as a protector of fixed calcium at the nucleation site. The idea has even been advanced that in calcareous algae photosynthesis is the driving force in carbonate deposition by consuming CO_2 molecules from an intracellular pool of bicarbonate.

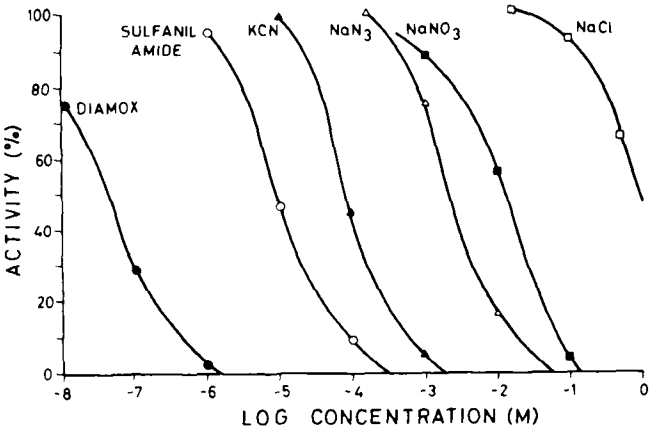


Fig. 31. Inhibition of carbonic anhydrase in a crude enzyme preparation from *Serraticardia maxima* by various compounds (after Okazaki[254])

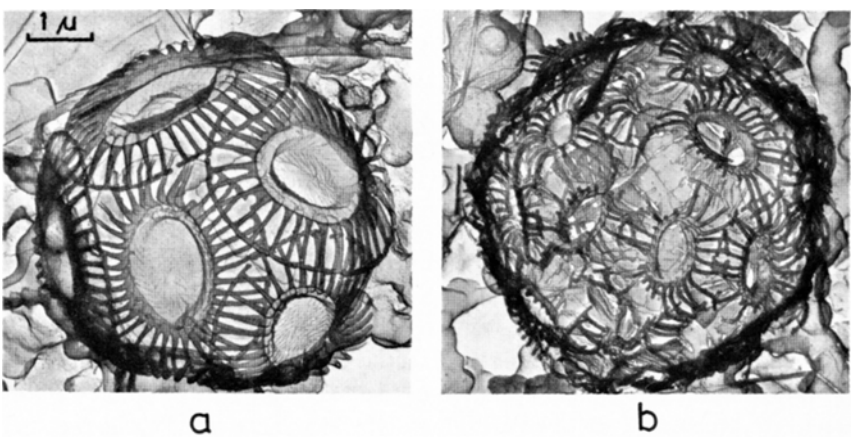


Fig. 32. (a) A coccolithophorid alga, *Emiliania huxleyi*, and (b) deformed coccolith from the same species. The deformed specimen was collected from the North Central Pacific. All specimens of *E. huxleyi* collected from this area were malformed like the one shown above. Natural environmental stresses such as abnormal temperatures, salinity or nutrient deficiency can cause such a pattern. Similar extensive malformation of coccoliths in vitro do develop by administering very dilute PCB's in the 200 ppt (part per trillion) range. The resistance to PCB is significantly different between coastal strein and off-shore strein even if they are the same species and morphologically identical. The coastal one is always more resistant (Honjo[262a]; Okada and Honjo, pers. comm.).

The strongest support for the involvement of carbonic anhydrase in the regulation of mineral deposition comes from studies on carbonic anhydrase inhibitors. In the presence of inhibitors such as Diamox or sulfanilamide (Fig. 31)[254–260] enzymic activities are strongly reduced and result in a decrease in shell thickness of eggs[261] or malformation in calcareous tests of the kind shown in Fig. 32[262]. In the presence

of carbonic anhydrase inhibitors, rates of calcification are strongly affected as shown in studies on corals[259, 263, 264].

These studies indicate that the actual role of carbonic anhydrase in calcification does not rest on its ability to hydrate CO_2 but to remove carbonic acid from the site of calcification:

$$Ca^{2+} + 2\,HCO_3^- \rightleftharpoons Ca(HCO_3)_2 \rightleftharpoons CaCO_3 + H_2CO_3$$

If corals are kept in darkness and in the presence of carbonic anhydrase inhibitors, calcification will still continue but at a much lower rate. The following conclusions can be drawn from these data:

Carbonate deposition involves interaction of Ca^{2+} and HCO_3^- resulting in the formation of an unstable intermediary product $Ca(HCO_3)_2$. This product will break down into $CaCO_3$ and carbonic acid (H_2CO_3). As long as calcium is not the limiting factor, the rate of formation of calcium carbonate will depend on the rate by which carbonic acid is removed from the calcification site. In the presence of carbonic anhydrase, calcification is significantly increased due to the formation of a complex between the high-pH form and a neutral H_2CO_3 molecule, which is the substrate for carbonic anhydrase[157].

Respiratory CO_2 is often mentioned as the sole or principal source for the carbonate ion in biological calcite or aragonite[265, 266]. Stable isotope data, however, argue strongly against this supposition[267–271]. Only in a few species can a significant contribution from respiratory CO_2 be demonstrated[272–274]. Most invertebrates and even the otoliths in fish[225] form their carbonate in or almost in isotopic equilibrium with the dissolved carbonate species present in their environment. In fact, the distribution of oxygen isotopes of the carbonates in many aquatic biological species reflects the water temperature in the habitat where the organisms live. Thus, we must assume that bicarbonate enters the inner system of the cell via permeable membranes where it forms a pool from which it is rapidly extracted leaving little possibility for $^{13}C/^{12}C$ exchange with the isotopically light respiratory CO_2. Alternately, carbonate deposition may proceed through direct contact with the outside environment.

Carbonic anhydrase seems to be not only a catalyst in calcification but also a vehicle for the translocation of reserve calcium. In the mantle of freshwater clams, for instance, the ratio of bound to free calcium ions is in the order of 10 to 1[275, 276]. A series of experiments revealed that the dynamic equilibrium between the pools of ionized and combined calcium is entirely controlled by carbonic anhydrase.

The enzyme functions as a kind of "chemical homeostat" by releasing calcium and carbonate to the extrapallial fluid from the reserves present in the mantle tissue. Because of the extreme sensitivity of the carbonate system to slight changes in pH (see p. 19, Fig. 14), carbonic anhydrase must operate in both directions in order to maintain a dynamic equilibrium between the bound and free calcium pools.

Calcium transfer observed in chick embryo may also be controlled by a similar mechanism since the newly hatched chick has six times the amount of calcium initially present in the egg yolk. In this case the egg shell is the bound Ca^{2+} pool. It is not clear to what extent calcium-activated ATPase or a calcium-binding protein carrier is involved in active transcellular transport of calcium[277, 278].

(vi) Functionality of Golgi Apparatus

Eukarotic cells contain complex membraneous organelles known as the Golgi apparatus or Golgi body which occupy a central position in the transport system of the cell. There is a wealth of information indicating that the Golgi is a key element in biomineralization. It is, for this reason, that we will look more closely into its functionality.

The Golgi complex is in a constant state of flux[279]. Cisternal membranes arising from the rough endoplasmic reticulum change to smooth endoplasmic reticulum and become the Golgi cisternae. Those cisternae then break down to vesicles which can fuse with and extend the plasmalemma.

At one surface the Golgi apparatus has a fresh face and at the opposite surface a mature face. Membranes at the fresh face resemble those of the endoplasmic reticulum, and membranes at the mature or secreting face are similar to the plasmalemma. This suggests that the Golgi apparatus operates as a membrane transformer from: endoplasmic reticulum → vesicles → Golgi apparatus → vesicles → plasmalemma.

This process appears to be irreversible. Vesicles derived from the Golgi cisternae have an inner mucopolysaccharide coat which – when transferred to the plasmalemma – appears at the outside of this membrane. Many other materials are transformed and packaged within the Golgi apparatus for export from the cell interior across the plasmalemma. These compounds include polysaccharides, glycoproteins, cations and anions. It appears that carbohydrate moities derived from the Golgi system is the essential "lubricant" for the transportation of material across the plasmalemma. One of the most complex functions of the Golgi apparatus is its role in calcification. As an example, the scale production mechanism in some brown algae is briefly outlined[280–293].

In coccolithophoridae the stepwise assembly of coccoliths which are composed of calcite[282] and organic matrix can be followed almost in slow motion from their point of origin in the cisternae of the Golgi system to their final resting site outside the cell membrane[283, 284]. Although one can recognize a species-specific development in the sense that some algae form the coccolith directly in the Golgi apparatus proper, while in others it is formed in special cisternas, they all share the characteristic that coccoliths are Golgi-elaborated structures[287]. The scales have a dorsoventral asymmetry and are oriented in a definite way within the coccolith-shaped vesicle. This suggests that the vesicle itself also has a dorsoventral asymmetry.

Removal of calcium carbonate from coccoliths will reveal an organic matrix with the outline of a coccolith[289]. Cells may also lose the capacity to produce coccoliths and only membraneous remnants may be found outside these so-called naked cells[287]. The organic matrix inside the coccoliths can become stained (Fig. 33), but different from the MM's in molluscs which is proteinaceous, the MM in coccoliths seems to be entirely composed of carbohydrates[222, 294]. In *Coccolithus huxleyi* uronic acid represent 25 per cent of the carbohydrate fraction and the rest are probably simple sugars and polysaccharide-sulfates[222a]. Ribose has been found in greater abundances in coccoliths[111]. Its presence may be linked to enzymic activities but more work is needed to pin-point its precise role.

High sugar concentrations have also been reported from the skeletal parts of some corals.

Fig. 33. Heavy-metal stained coccolith, *Umbilicosphaera sp.*, revealing growth pattern

In carrageenans which are polysaccharide sulfates present for instance, in human cartilage and plant cell walls, the tertiary structure has been determined by means of X-ray diffraction analysis[295]. A double helix structure was proposed with a hexagonal array of helices of diameter about 13 Å. In each chemically distinct polysaccharide chain there are three disaccharides on one turn of a helix with a repeating distance of 26.0 Å for iota-carrageenan and 24.6 Å for kappa-carrageenan. The hexagonal array of double helices (Fig. 34) is held together by interactions between the sulfate groups and cations.

In their natural state both carbohydrates occur as a gel; cations are required for gelation and two or more chains can be cross-linked (Fig. 35). Thus, they function not only structurally but act as a carrier of metal ions and water. Cations are coordinated to oxygens in sugar, sulfate, and possibly water. The resulting metal ion coordination polyhedra of the type occuring in DNA (see Fig. 3) provide a double backbone for the stabilisation of the structure. Other polysaccharide networks, of animal and seaweed origin, have primary structures based on an alternating arrangement of 1,3 and 1.4 linkages. Similar conformation principles as in carrageenans may exist. A triple helix has been proposed for an algal xylan. Actually, whether the carbohydrates in question occur as single-, double- or triple-stranded helices is most likely determined by type of metal ion that coordinates to the carbohydrate. This is in line with conformational changes introduced in nucleic acids with metal ion substitutions[53, 54].

It is conceivable that a polysaccharide sulfate acts as the substrate in the mineralization of a protozoan group, the acantharid radiolaria, that deposit a celestite ($SrSO_4$) skeleton with small amounts of barite ($BaSO_4$) in solid solution[295]. Other polysaccharides are known with carbonate groups and they are likely candidates in carbonate deposition. Thus, a number of polysaccharides exist that can selectively

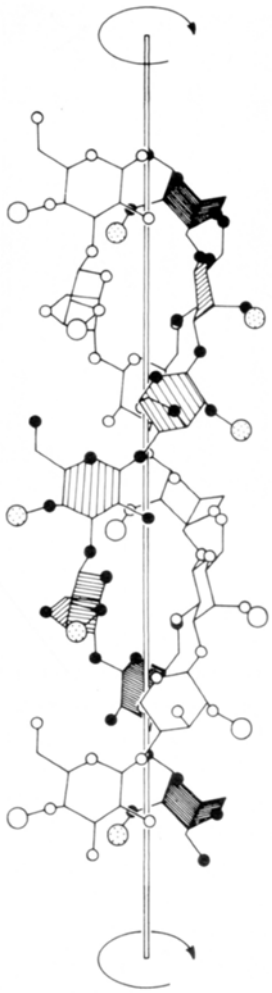


Fig. 34. Model for the ioata double helix. Open circles represent oxygen atoms and larger (dotted) circles SO_3-groups (after Anderson *et al.*[295])

chose metal ions and acquire a distinct conformation which in turn determines the mineral form.

The presence of sugars and uronic acids on the inner walls of the Golgi cisternae may provide sites for the coordination of calcium which is ultimately derived from the endoplasmic reticulum. The fluids inside the vesicles may thus represent the functional analogue to the extrapallial fluids in molluscs[45]. Changes in pH will effect the solubility of $CaCO_3$ and may result in the deposition of calcite. Carbonic anhydrase appears to be catalytically involved in the reaction, since a treatment with inhibitors of this enzyme leads to morphologically deformed or underdeveloped scales of the kind shown in Fig. 32. During expulsion of the coccoliths from the Golgi apparatus the scales become turned to the outside.

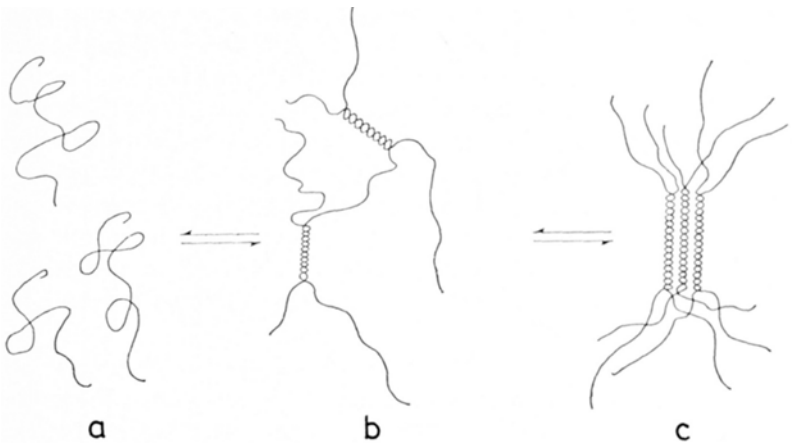


Fig. 35. Suggested relation of polysaccharide chain (a) in solution and (b) and (c) in the gel (after Anderson *et al.*[295])

The Golgi membraneous system also occupies a central position in mollusc calcification[297]. However, different from the coccolithophoridae, the actual $CaCO_3$ deposition does not take place inside the Golgi vesicles which are abundant in epithelial cells lining the surface of the outer mantle fold. Instead, the calcification site is an organic matrix released from the extrapallial fluid separating the mantle from the shell.

In addition to its role in carbonate deposition, the mantle is also connected with other physiological activities such as secretion of mucus or the formation of the periostracum[298–304]. Certain regions of the outer mantle surface are allocated to various work assignments and differ in their ultrastructural composition.

Golgi elements in the shell-secreting epithelia of the mantle edge rapidly increase in size prior to calcification and secretory granules come into existence. Here, the glycoproteins are assembled. The Golgi secretory product can occasionally be observed in the layer of fibrillar material present at the cell surface[297]. This layer may represent a polymerized organic matrix which probably is the carrier protein (CP). The CP will interact with the soluble glycoprotein complex released from the Golgi secretory granules. The complex will be split into a carbohydrate and protein fraction and the latter becomes attached to the CP. The presence of large quantities of glycogen granules in the immediate vicinity of carbonate deposition sites may be derived from the carbohydrate fraction which formerly had served as the carrier of calcium and the mineralizing matrix. At present the role of lysosomes and enzymes in this part of the calcification mechanism is not fully understood. The abundance of alkaline phosphatase in this strategic region of the mantle implies an involvement of this enzyme in the synthesis of fibrous proteins and mucopolysaccharides, as well as calcium transport and regulation[305].

Concerning the source of calcium, cytochemical studies on its distribution in the mantle epithelium are quite revealing[306]. It appears that calcium is present in free and combined forms. Free calcium migrates from the endoplasmic reticulum

from where it is picked up by the Golgi vesicles. The mode of association inside the Golgi membraneous system is still uncertain. If we take the coccolithophoridae as a reference, a mucopolysaccharide coat serves as a template in calcium fixation in molluscs too. Alternately a mineral phase may well exist within the Golgi vesicles. Migration of the vesicles and their opening to the extrapallial space releases the calcium carrying substrate which then transfers its calcium to the freshly polymerized MM. This process is comparable to the action of troponin on the actin filaments during muscle contraction in vertebrates or of myosin light chains in molluscs. A coordination change is all that is involved. To what extent free calcium present in the extrapallial fluids participates in this reaction cannot now be evaluated. Most probably this depends on the $CaCO_3$ equilibrium.

The distribution pattern of Golgi elements on mantle epithelia gives rise to spiral or reticulate thickening of the wall. This in turn controlls the orientation of the individual $CaCO_3$ crystals which in the case of the nacreous layer of molluscs may be arranged in the form of spirals. The Golgi distribution pattern also effects the ratio between organic and inorganic phases.

The fact that coccolithophoridae deposit their $CaCO_3$ inside the Golgi vesicles may be fortuitous. We should remember that calcification is a rather late development in the evolution of brown algae and may be linked to the chemical evolution in the sea.

The same could be true for molluscs, except that here calcification started at an earlier geologic age. From what we have presented about the mechanism of calcium carbonate deposition and its close association with the activities of the Golgi apparatus, one cannot escape the conclusion that the whole operation was originally conceived only for the rapid and efficient removal of calcium and perhaps some other elements from within the cell. The unique role of calcium in so many physiological activities[307] must also allow for a valve system for the discharge of unwanted surplus. Mineralization probably started as an "accident" but once a reality it became explored by the organisms.

In the case of some prokaryots which lack a Golgi apparatus, calcium carbonate deposition is an extracellular process[308,309]. In stromatolite communities which are represented by blue green algae and bacteria, formation of carbonates is entirely environmentally controlled. The organisms alter the chemistry of its next environs by releasing ammonia, phosphates, carbon dioxide and dissolved organic matter to facilitate the formation of crystalline phases. Control of crystal form is most likely related to the type of metabolic products discharged by the organism and its effect on pH. Bacterial precipitates can involve intermediate phases such as monohydrocalcite which is rare in contemporous environments[310,311]. They subsequently may become altered to a stable phase such as calcite. Thus, bacteria and blue green algae due to their metabolic activities, generate a microenvironment which promotes deposition of $CaCO_3$ and other minerals such as struvite and high-magnesium calcite even though the macroenvironment is unfavorable for $CaCO_3$ precipitation (Fig. 36[312]).

Stromatolites are known for more than 3 billion years[313–315], whereas the first shell-forming Metazoa appeared 600 Million years ago, when the sea may have become saturated with respect to $CaCO_3$. Nannofossils represent an even later de-

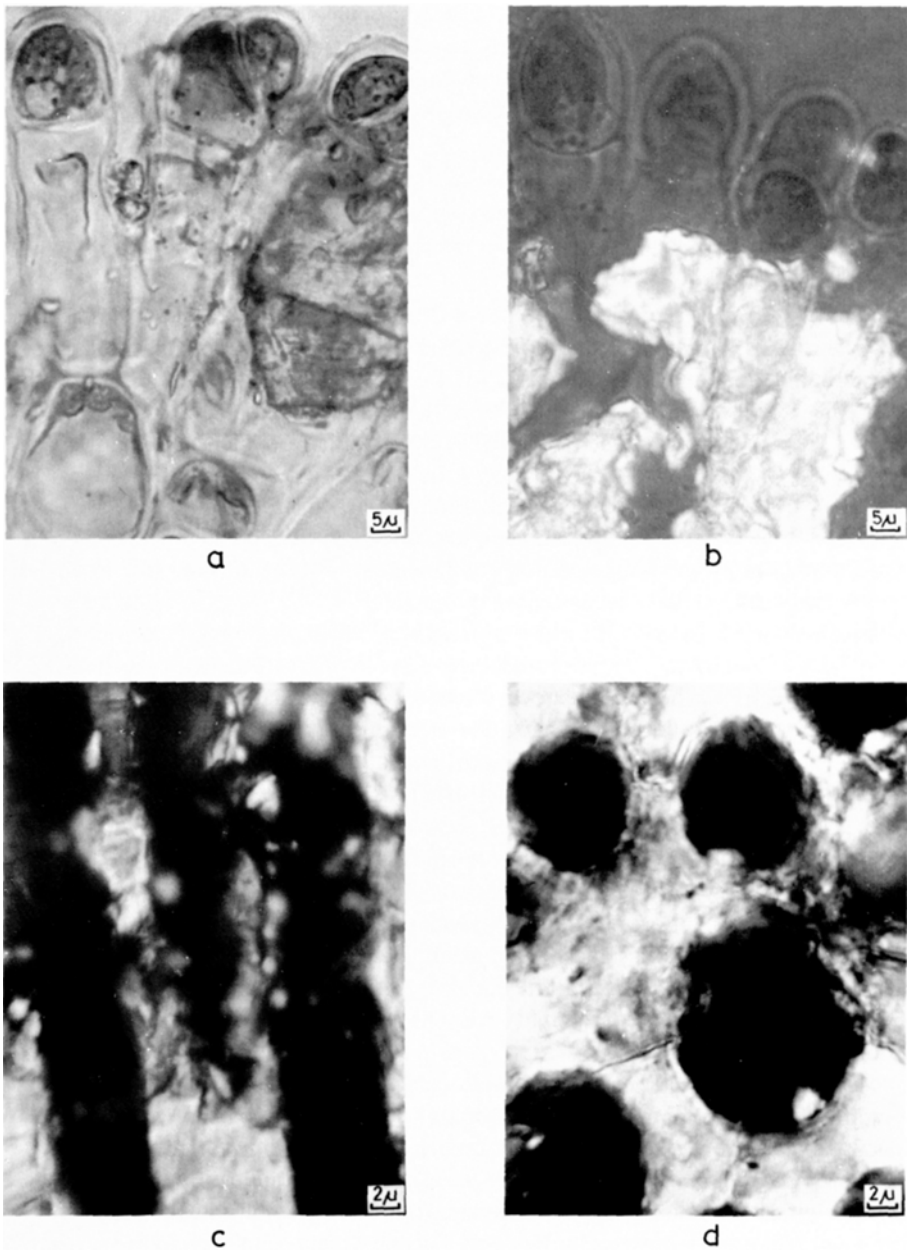


Fig. 36. (a) *Gongrosira incrustans* (Chaetophorales, Chlorophyta). Surface of an algal thallus. Terminal cells of algal filaments contain most of the chlorophyll. Photomicrograph, transmitted light; (b) surface of a calcified thallus; semipolarized light; (c) the interior of a calcified thallus; longitudinal section. Calcite crystals arranged tightly around algal filaments, (polarized light); (d) the interior of a calcified thallus; cross section. The shape of calcite crystals conforms with the size and arrangement of algal filaments (polarized light) (after Golubić and Fischer[309])

velopment, occuring for the first time in the Mesozoic. Perhaps the calcium level in the sea or its availability to organisms was the controlling device for the "retrograde" calcification pattern which started from the extracellular, moved to the extrapallial, and finally reached the vesicular stage inside the Golgi membraneous system.

(vii) Skeletal Growth Rate

Studies on growth patterns of carbonate depositers has revealed striking similarities in organisms as distant as corals, molluscs and fishes. This suggests that there is some common denominator. Most characteristic is a distinct banding representing growth stages in the development of the organism. By means of radiographic techniques as well as simple counting of the individual bands it could be shown that banding can represent annual, monthly or daily growth increments or can be caused by tidal effects or other perturbations in the environment. Band widths allows precise determination of the amount of carbonate deposited or not deposited within a given time interval or by a certain environmental incident. This is not the place to critically review the wealth of data accumulated in this field[263,264,316–333]. Only a few observations are listed which throw some light on the physiology of calcification.

The idea has frequently been advanced that calcium carbonate deposition in plants is directly connected to carbon dioxide withdrawal and hydroxyl ion production during photosynthesis. In reality, the mechanism is more complicated. It was shown that calcareous algae, except for the coccolithophoridae, maintain high rates of calcification even in the dark[264, 334]. The most probable explanation is daytime formation of a reservoir containing the whole suite of calcifying agents which during the night are gradually extracted. The nature of these agents is not known, but may involve high energy organic materials. It was surprising to find that reef-building corals can become "photosynthetic" because of a symbiotic relationship with algae. Calcification rates in corals associated with such algae are almost an order of magnitude higher in the light than in the dark[328]; and in coral communities free of symbiotic algae rates are considerably reduced and exhibit no diurnal fluctuations[259].

In studying a coralline algae, *Bossiella orbigniana*, and a reef-building coral, *Acropora cervicornis*, striking similarities in the calcification pattern were recognized[328,329]: (1) the tip of each coral branch is less heavily calcified than more basal sections, (2) rates in calcification decrease from the tip basally, and (3) in light, calcification rate is greatest in the tip. By isolating coral tips, the effect of ligth on calcification was greatly reduced. This observation implies that the flow of photosynthetic products derived from symbiotic algae is critical for fast deposition of $CaCO_3$ in corals[320]. In conclusion, a calcifying agent of a symbiotic plant can become grafted onto a coral and enhance its rate of calcification. Such vital effects may also account for the wide isotopic variability in coral and algal carbonates. In view of the similarity in amino acid pattern in calcareous algae, reef-building corals and oolites it seems very probable that oolites too have derived their calcifying matrix from a coexisting algal source[109–111].

Corals such as *Montastrea annularia* and *Madracis mirabilis* have a steady growth rate throughout the year if environmental parameters remain fairly constant and symbiotic algae are virtually absent[317]. The monthly weight increments amount to about 1 and 5 per cent, respectively. Annual gross production of a reef community ranges from 4×10^2 to 6×10^4 g $CaCO_3/m^2$ [335].

A significant aspect of skeletal growth in corals is the existence of an internal calendar where daily, seasonal, and annual records are kept on file in the form of individual or series of bands. The band thicknesses within a species are environmentally controlled. The number of bands per year in fossil corals has been used in studies on the Earth-Moon system, *i.e.* effect of tidal friction on number of days per year[336].

In a study on the impact of sedimentation on coral growth it could be shown that corals can free themselves from sediment material with the extrusion of much mucus[322]. During such an event coral growth is greatly inhibited and variability in growth decreases. Apparently the energy expenditures for removal of the sediment particles and the utilisation of calcium for muscle or energy transfer has left no excess calcium for biomineralization. This again underscores the intimate relationship between the various calcium-requiring systems in the cell. During an active stage little if any calcium is exported to the cell periphery and consequently calcification is at a minimum.

Microscopic growth increments 1 to 100 microns thick have been recorded from mollusc shells[327]. These were explained as daily deposits. However, a note of caution should be sounded in view of the findings that in some shells tidal growth increments are indicated and that the average duration of an increment is 24 hours and 50 minutes (circalunadian rhythm)[337].

During exposure at low tide growth stoppage apparently does occur giving rise to a sharp line composed of organic matrix. The relationships of growth pattern to environmental stress have been examined by transplanting juvenile specimens of *Mercenaria mercenaria* from holding tanks to various environmental settings[327]. Growth rate decreased in intertidal zones and at the surface of a turbid subtidal mud bottom. Calcification rates were higher in specimens living in sand than in mud. Specimens elevated about half a meter above the muddy bottom showed accelerated growth.

In a related experiments, shallow and deep water forms were compared[327]. In deep water specimens increments had a uniform thickness, without sharp boundaries and no apparent seasonal or tidal indications. In summary microgrowth patterns reflect environmental changes of many sorts. The ultimate reason behind this phenomenon can be explained on physiological grounds as a result of the utilisation of calcium in energy transfer and muscle activities. Namely at times of stress these activities have higher priorities than calcification.

Periodical patterns of a similar kind are recorded in fish otoliths which seem to participate in the fish auditory system[338, 339]. Otoliths are composed of alternating layers of organic matrix and aragonitic prisms[225]. The corresponding dark and light bands run approximately parallel to the outer surface of the otolith (Fig. 37). Aragonite needles are oriented with their *c* axis perpendicular to the bands or their projected surface respectively. They extend from the center to the margin of the otolith without being physically interrupted by the dark organic layers. The band pattern is interpreted to represent annual or seasonal growth rings which allow dating of the fish.

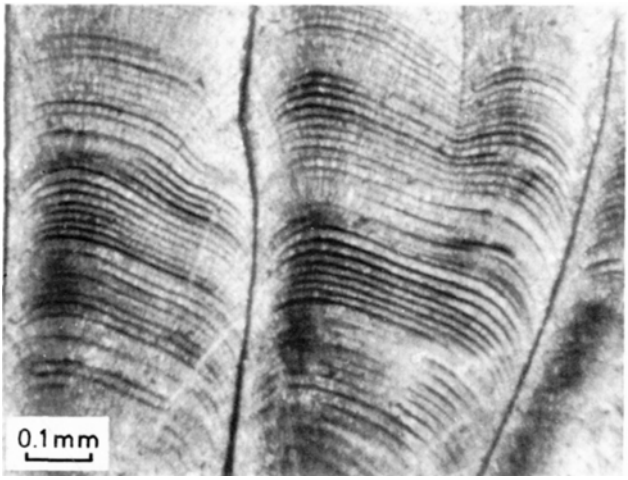


Fig. 37. Otolith thin-section of *Roccus lineatus* approximately parallel to the lenticular faces (after Degens *et. al.*[225])

Banding is most intense in the early-stage annual rings and becomes less pronounced as the fish grows older.

Closer examination of the otolith fabric has revealed zones of fast and slow growth[321]. Each zone is characterized by alternating light and dark colored microlayers. During fast deposition, production of organic matrix is high but calcification is even higher, whereas at times of slow deposition calcification is almost nil and the observed increments are due to organic matter contribution. The slow growth zone is composed of 100 to 110 bands and the fast growth zone of 230 to 260 bands which represent winter and summer layers respectively. In addition, there are band series of weekly, fortnightly and monthly periodicities. This indicates that growth takes place by daily increments which are most likely related to known circadian rhythms in the vital responses of fishes.

Aragonite crystals reflect this routine daily calcification in the form of a wedge-shaped pattern (Fig. 38 a, b) which is lacking in aragonite crystals which are precipitated inorganically[340]. Up to 15 growth interruptions per micron can be counted which adds up to several thousand individual growth segments across a single aragonite needle extending from center to margin of the otolith. The explanation for such a fabric may be found in the chemistry of otoliths which shows sodium concentration of 2 mole per cent relative to calcium equal 100; sodium may interfere with regular growth of aragonite. Isomorphous substitution of sodium for calcium in aragonite is unlikely. Instead it has been proposed that an accessory mineral such as shortite $Na_2Ca_2(CO_3)_3$ or pirssonite $Na_2Ca_2(CO_3)_2 \cdot 2\,H_2O$ coexists with the aragonite[337]. Both these minerals are orthorhombic as is aragonite and wedge-shaped crystals are the typical habitus of shortite. Sodium ions could interfere with the growth of aragonite by either occupying coordination sites on the organic matter or by epitaxial attachment of shortite on aragonite.

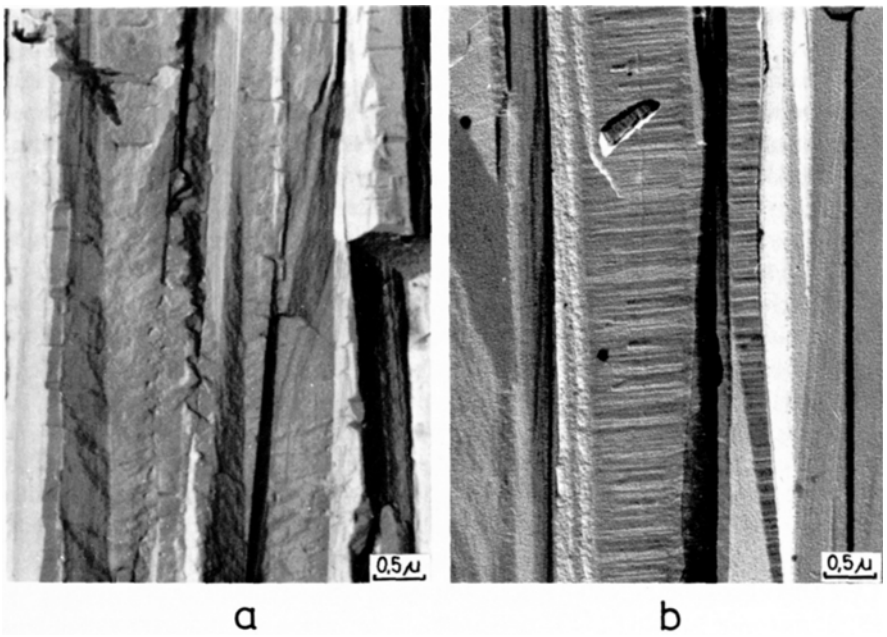


Fig. 38. (a) Electronmicrograph (Pt/C replica) of otolith of *Aplodinotus grunniens;* (b) inorganically precipitated aragonite (Pt/C replica) (after Degens *et al.*[225]; Hathaway and Degens[340])

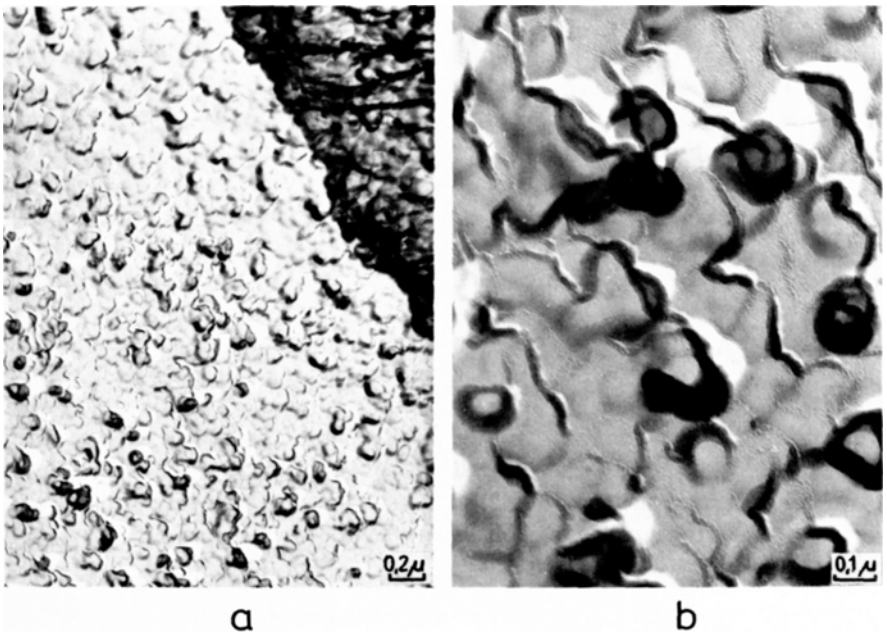


Fig. 39. (a) Electronmicrograph (Pt/C replica) of otolith of *Aplodinotus grunniens* showing the surface structure of the mineralized tissue; (b) same enlarged, showing coiled protein chains (after Degens *et al.*[225])

Sodium or one of the proposed trace minerals would act in the capacity of a growth inhibitor for aragonite. A new increment of $CaCO_3$ will only form following the deposition of an organic template.

The organic matrix in otoliths is quite uniform with regard to amino acid composition in spite of its wide concentration range of 0.2 to 10 weight per cent of the total. The MM is a fibrous protein with a molecular weight greater 150000. Employing hydrogen bond breakers the protein will degrade to units of 70000 to 80000 MW[225]; its amino acid composition is shown in Table 3. The fibrous protein is concentrated in the dark-colored bands and oriented in a kind of corrugated pattern with well defined lineages; the fibers are composed of individual chains organized in a helical fashion (Fig. 39 a, b). At certain intervals the chains are twisted to such an extent that lumps or knots of "apparently tangeled" fibers appear about 0.1 to 0.3 micron apart. The cross section of an individual fiber measures about 100 Å.

In contrast to molluscs where a carrier protein and a mineralizing matrix are needed for carbonate deposition, fish otoliths contain only one specific protein. It is, therefore, possible that part of the regulating mechanism is provided for by sodium ions. They possibly become coordinated to sites normally occupied by calcium ions and in this way block further aragonite growth. Similarly the high amount of fluorine content of some foraminiferal tests may be used to terminate calcite growth by substituting for oxygen in $Ca^{2+}O_6$ polyhedra. In any event, calcium carbonate formation requires deposition of a new MM blanket. Thick organic layers in otoliths mark incidents unfavorable to carbonate growth. They appear to be associated with some special stress conditions in the lifetime of the fishes such as spawning or breeding.

Although only a few examples were discussed, these examples are rather characteristic for skeletal growth patterns in calcareous organism. We could demonstrate that carbonate deposition is in many ways a clock-controlled biological rhythm. The effect of solar day, lunar day, season or year, can be recognized in the form of specific criteria such as bands, layers and other growth patterns.

The biological clock may be a feedback system involving ions and ion-transport channels. A model has been proposed[341] where membranes introduce configurational changes in response to oscillating ion concentrations whose frequency has been set by a series of environmental parameters such as light, temperature, and tides.

Superimposed on these records are markings of environmental perturbations which sometimes inhibit, and sometimes accelerate carbonate deposition. In the final analysis, however, there has to be an answer, why organisms respond in such a systematic fashion. In all probability these phenomena are connected to the high demand for calcium at all levels of the cell regulatory system. Only at times of lower activities or when the calcium pool runs over will the excretory system package and export the surplus calcium to the periphery of the cell where it may be utilized in carbonate deposition.

(viii) Evolution of Calcified Tissue

The best approach to understand the evolution of calcified tissues would be a sequence study on polymers such as proteins and polypeptides present in shell struc-

tures. By using statistical methods, this information could lead to the construction of a phylogenetic tree[342]. Unfortunately, such studies have not yet been made largely because of the heterogenous nature of the mineralized tissue which first must be fractionated into its individual polymers prior to sequence determinations. Inasmuch as isolation of pure polymers is beset by too many analytical difficulties, several research groups have followed a different approach. They hypothesised that the shell matrix was derived from the gradual evolution of at least one structural protein which originally had some function other than which it now serves. With the change from a non-calcified to a calcified state, the protein became increasingly reorganized so as to facilitate the effective binding of calcium. In general, this should involve a proportionate decrease in the quantity of superflous organic materials.

Based on this rationale the shell organic matrix from a wide selection of species was analyzed for amino acid and sugar contents[217, 218]. At first sight the results turned out to be unexpectedly complex, but a closer examination revealed a common pattern.

Each species contained a different spectrum of amino acids and sugars which for the purpose of comparative biochemistry represents an excellent start. However, in trying to describe and understand the complex biochemical interrelationships among amino acids and sugars of the heterogeneous matrix, and to outline phylogenetic relationships factor analysis techniques were necessary. Factor analysis is a generic term for a variety of techniques that seek to explain complex relationships among many variables in terms of simpler relationships among fewer variables. For molluscs, four to five major factors described the relationships of the amino acid and amino sugar distribution[217, 218]. For a series of planktonic foraminifera where only the dominant amino acids were processed, three independent factors could explain the observed variability[213, 214]. On the basis of factor analysis it is now possible to construct phylogenetic trees which closely conform with trees based on classical biological criteria such as morphology.

Factor analysis is just emerging as a powerful tool in comparative biochemistry of calcified tissues. There is clear-cut evidence that various lineages have undergone the same changes repeatedly and independently. Because of mosaic evolution the rates at which changes occur in a single lineage are not necessarily the same for different structures. Convergent changes occur in freshwater and terrestrial environments. Amino acid patterns in the shells of some highly evolved land snails resemble that in some ancestoral marine forms. It is probable that a shift to a new habitat made it advantageous to maintain the same functional units which had been decreased in proportion among advanced marine forms. Temperature and salinity effects are recorded which are characteristic for each species and not for a larger taxa as a whole. It should again be emphasized that molluscs taken from the same environment exhibit a species-specific amino acid pattern. For other forms data are less plentiful but similar trends are discernable.

From a structural point of view it is unlikely that a protein responsible for such a specific duty will undergo severe modifications in the course of evolution.

Evolving proteins in general may of course change some of their amino acids but only at sites which are not crucial for the maintenance of their particular functions. Thus, the MM which is largely composed of acidic amino acids represents a

rather stable element in the organic matrix of shell structures. For example, in metazoan calcification, aspartic acid is the most essential amino acid. The observed fluctuations in amino acids are largely due to carrier proteins which are secreted in various proportions and rates. The water soluble glycoprotein found as intracrystalline matrix in shell carbonates is another contributing factor[228]. Its presence may be linked to the transport of calcium from mantle to MM and its inclusion by the growing carbonate crystals.

The amino acid composition of MM resembles that of troponin[199] and the light chain of myosin[192]. Thus it seems probable that these proteins are homologous and that the same conformational principles apply[200]. For instance, troponin binds calcium at all four Ca-bindung sites in octahedral coordination[203]. In contrast, the MM can tie calcium in either $Ca^{2+}O_6$ or $Ca^{2+}O_9$ coordination polyhedra[343]. When calcium becomes six-fold coordinated to MM, the epitaxial outgrowth is calcite; nine-fold coordination yields aragonite. Magnesium can substitute for calcium in calcite, and strontium may proxy for calcium in aragonite. The degree of isomorphism depends on several factors of which three important ones are: (1) phylogenetic level[344], (2) water temperature[345], and (3) salinity[346]. In view of the fact that some organisms, *e.g. Mytilus* may alternately deposit calcite or aragonite depending on water temperature[210] can best be understood by the ability of MM to adjust to temperature fluctuations through conformational changes thereby accomodating calcium in the best possible way.

The key role exercised by aspartic acid in MM is probably taken over by serine in the carrier protein. Conformation of the CP under ideal conditions involves only β-pleated sheets but, depending on the primary structure, α-conformations will occur. The ratio of glycine: alanine: serine largely determines which types of cross-β-structures are developed.

Also, increase in water temperature favors β-conformation. Inasmuch as the conformation of CP probably determines the tertiary structure of MM, slight changes in CP conformation introduced by cellular or environmental effects may alter MM conformation. This in turn is reflected in the mineral form and structural pattern of the inorganic phases. Perhaps, nacreous layers in molluscs represent an almost ideal situation where MM and CP are aligned in a symmetrical way. In fish otoliths, the fibrous organic matrix is a mixture of helices and β-pleated sheets. It is tentatively concluded that the morphology of shell structures is a macroscopic expression of the molecular interactions between MM and CP which are controlled in part by cellular activities and in part by the environment.

We further conclude that calcified tissues were not developed for carbonate deposition. They were secreted as components of the cell wall or as excretory by-products of metabolic processes. As a consequence of a changing habitat, these materials started to calcify or introduced inorganic carbonate deposition. The sudden occurence of highly developed metazoan populations at the boundary Cambrian-Precambrian is frequently explained in terms of an explosive evolution[347]. For those who are familiar with the mechanism of calcification this "deus ex machina" explanation is not necessarily the most satisfying one. It may simply be a reflection of the universal adaption of many forms of life to a new environmental setting that developed at that time. Only a slight modification in pH is required (see Fig. 14) to

dramatically effect the solubility product of $CaCO_3$. In addition, increase in oxygen content in atmosphere and hydrosphere may have reached a critical level close to the Precambrian-Cambrian boundary and allowed organisms to turn on at full speed the advantageous oxidative metabolism. "Body-building" processes were the consequence and caused a high demand for Ca^{2+} ions. Mechanisms had to be designed to channel calcium ions in and out of the cell. Some organisms which secreted their excess calcium via the Golgi apparatus into the open sea across the plasmalemma suddenly started to calcify. Others remained uncalcified since they used all cellular calcium for energy expenditures or muscle activities. The fact that, even today, many normally calcifying organisms stop $CaCO_3$ deposition at times of environmental stress or during active stages may serve as a reminder that calcification ranks in low priority for the calcium metabolism of the organism. Furthermore, many advanced forms that are descendants of shell-depositing ancestors have lost the ability to mineralize; most commonly, they represent highly mobile and energetic varieties. In contrast, heavily calcified organisms are, as a rule, the more dormant representatives.

VIII. Phosphate Deposition

(i) Mineral Phase

The nature of mineral phases present in bone, dentin, enamel and other phosphatic tissues, and their mode of formation have been subjects of lively discussions among health scientists and crystallographers. Bioscientists most commonly accept the viewpoint that the inorganic phase of bones or teeth is principally hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, and deviation in Ca/P ratio from common hydroxyapatite (Ca/P = 1.667) observed in mineralized tissues is explained by the presence of amorphous phosphates. In contrast, many crystallographers favor the idea of carbonate apatite, *i.e.* dahllite, as the major crystalline phase in biophosphates and they doubt the existence of amorphous phases. The topic has been reviewed[14, 15, 22, 28, 37, 44, 47, 348–358] but no common consent has yet been reached. In the following an attempt is made to at least coordinate the controversial findings.

Crystals in bone, dentin and enamel can vary greatly in size and in shape[359–361]. For example, the bulk of the apatitic crystals in young bone material is small and compact having a mean cross-sectional width of only about 90 Å[354]; in addition one will occasionally encounter larger crystals in the 500 to 1000 Å range which have a thin platelet-like form. It is of note that the two crystal populations exhibit no continuous progression of sizes and are morphologically distinct from one another. Since size and form of crystals are a function of a number of environmental parameters, the two species in question must have originated under somewhat different conditions.

Crystals showing a regular hexagonal outline in cross section and having straight, parallel edges when seen in longitudinal section are rare in normal calcified tissues but occur in hypermineralized surface regions of cervical cementum and root dentin (Fig. 40)[358]. Under the electron microscope individual crystals exhibit a minute

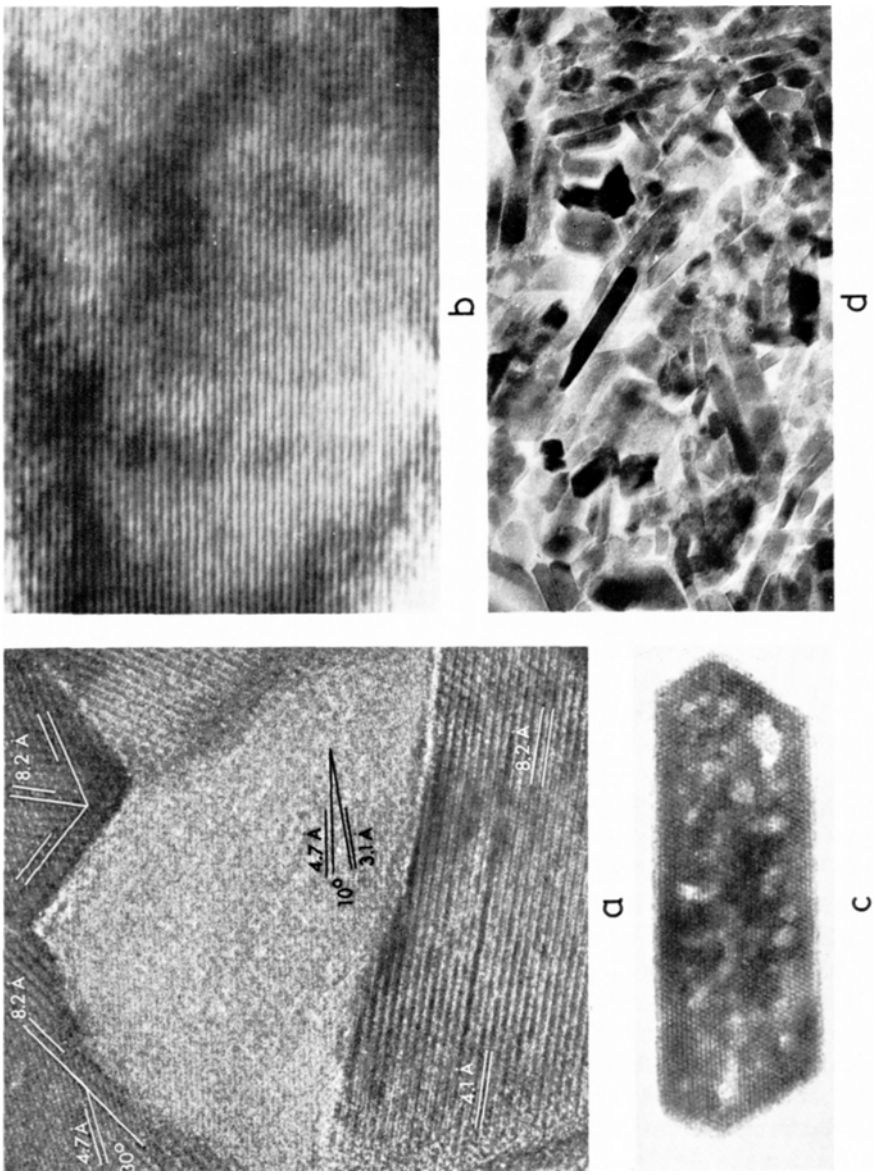


Fig. 40. (a) Electronmicrograph of enamel showing lattice fringe patterns in individual apatite crystals; (b) regularity of repeating pattern; (c) apatite crystal in hypermineralized dentin; slightly damaged by the electron beam; (d) crystals in hypermineralized dentin showing varies sizes and shapes (Selvig[359–361])

Table 6. Diffracting planes in the hydroxyapatite crystal lattice demonstrated by electron microscopy[362]

Indices *hkl*	Spacing *d* Å	Observed spacing
100	8.17	8.2
101	5.26	5.3
110	4.72	4.7
200	4.07	4.1
111	3.88	Not observed
201	3.51	Not observed
002	3.44	3.4
102	3.17	Not observed
210	3.08	3.1
211	2.81	Not observed

striation pattern in the form of alternating light and dense bands which represent the resolution of sets of lattice planes. Cross-sectioned crystals reveal a 8.2 Å repeat period which well conforms with the (100) planes of the ordinary hydroxyapatite crystal lattice (8.17 Å)[360]. Other spacings and directions can be resolved under the electron microscope which conform with projection planes of hydroxyapatite[362]. Thus, within certain limits, calculated spacings and angles of intersection of major lattice planes in ordinary apatite correspond with observed lattice fringe patterns of the type seen in Fig. 40. For illustration, the *d* spacing of lattice planes in the hydroxyapatite crystal lattice[362] and the spacing measured under the electron microscope are presented in Table 6. The two sets of data correspond closely to one another; some lattice planes could not be measured because they were obliquely oriented in relation to the crystal axes.

In enamel and hypermineralized dentin more than 100 repeat periods were counted across one single crystal. However, in normal bone and dentin, individual crystals show fewer repeats, commonly less than 10 and as little as 4; this would correspond to a cross-sectional width of about 40 to 90 Å[360]. The length of the crystals is in the range of a few 100 Å. In summary, at least two morphologically distinct types of crystallites are present in bones and teeth, and the smaller-sized fraction represents the bulk of the mineral phase.

X-ray or electron diffraction analyses are commonly employed to determine mineral species. In the case of biophosphates, these techniques are limited because the material is a complex mixture of organics and inorganics and furthermore the crystallites are small. Thus, resolution of X-ray diffraction pattern of bone and dentin material is rather poor. Electron diffraction pattern is generally better, but there is always the possibility of secondary alteration of the specimen during exposure. Other methods – such as infrared analysis – have their limitations too. In short, there are some analytical problems which may in part account for the conflicting interpretations offered in the literature.

The most straight-forward possibility is one in which the mineral phase consists only of dahllite and the presence of amorphous inorganic solids is excluded[28, 356, 363].

Variation in Ca/*P* ratio is explained by isomorphous substitution of calcium by elements such as sodium or magnesium, and exchange of tetrahedral PO_4 groups by planar CO_3 groups. The incorporation of water in the form of H_2O, H_3O, H_4O_4 and resulting structural adjustments are listed as an additional factor in controlling Ca/P ratio in so-called "hydrated carbonate apatites". The measured Ca/P ratios for hydrated carbonate apatites of mineral and synthetic origins cover a range from 1.53 to 1.87[363], which is about the range observed in biophosphates (1.33 to ~2)[359]. X-ray and electron diffraction data are listed in support of this inference. The results clearly show that enamel, dentin, and bone mineral cannot be equated with $Ca_{10}(PO_4)_6(OH)_2$. However, the data do not convincingly prove that the concept of dahllite as the principal or exclusive mineral phase in biophosphates in correct.

In a detailed and careful comparative study on the crystallography of bones from young and adult humans[354] it was demonstrated that bone minerals are not monomineralic but represent a mixture of several structurally different crystalline phases.

The principal mineral species is an apatite which is best defined as an isomorphous mixture consisting of two endmembers: hydroxyapatite and carbonate apatite, *i.e.* $Ca_{10}(PO_4)_6(OH)_2$ and $(Na, Ca)_{10}(PO_4, CO_3)_6(OH)_2$. Because of the isomorphous nature of the material the proportions of CO_3 that substitute for PO_4 most certainly vary from one unit cell of bone mineral to another and no uniform composition should actually exist. The extent to which carbonate groups proxy for phosphate groups in the crystal lattice is in the order of a few to a maximal of 10% by weight.

In addition to the wide spectrum of apatites, young bones contain calcium monohydrogen phosphate dihydrate (= brushite) with the compositional formula: $CaHPO_4 \cdot 2\ H_2O$[354]. It has been suggested that brushite is not stable above 25 °C unless it is in contact with a liquid phase[28]. Hydrolytic transformation will readily alter brushite into hydroxyapatite[354], and at a pH of 6.3 and temperatures around 40 °C formation of octacalcium phosphate has been observed. Since specimens are commonly subjected to a heat treatment (105 °C) prior to analysis it is not surprising that brushite in young bones has escaped detection for a long time. The mineral has, however, been reported from dental calculus.

A further mineral phase known from young bones and dentin is octacalcium phosphate for which no mineral name has so far been given[364]. The formula is: $Ca_8H_2(PO_4)_6 \cdot 5\ H_2O$. Octacalcium phosphate is composed of two layers which are oriented parallel (001). One of the layers is apatite and the other is water. The Ca/P ratio is 1.33 instead of the theoretical value of 1.667, because some lattice positions for calcium are vacant (= Ca-deficient structures). Octacalcium phosphate has been mentioned as a possible precursor of the small apatite crystallites in bones and teeth[358, 365–370]. Indeed, boiling water or fluorine-containing solutions will readily hydrolyze the material and convert it to hydroxyapatite. Fluorine is an excellent catalyst in this conversion process and its therapeutical value rests on this action[371]. In this context it is of interest that the shell of the inarticulate brachiopod genus *Lingula* is composed of francolite[372] (carbonate fluorapatite).

Whereas octacalcium phosphate has been found as a minor constituent in young bones, the claim has been made that the main crystalline phase in bones is octacalcium phosphate carbonate[373]. This suggestion is certainly not correct, yet, one aspect of the study is quite significant in that attention is drawn to the possibility

that the crystalline material in biophosphates is not anhydrous – "bone dry" – but hydrous in nature. Little data exist on the amount and structural position of water in the apatite lattice, and on the hydration state of metastable phases. Information on the biogeochemistry of water in biophosphates is urgently needed.

The subject of amorphous phases in biophosphates is even more controversial than the nature of the crystalline phases. One source of information relegates amorphous phases to the "realm of the mystics"[28] p. 75, whereas other sources voice strong opinion in favor of amorphous phases[374, 375]. Experimental evidence is presented suggesting that as much as 40 per cent of mineral matter in bone is amorphous calcium phosphate (ACP). Electron microscopic examination of dried ACP reveals discoidal and spherical forms[374] but it is questionable whether these two forms actually exist in living bone; it is more likely that they are produced by drying or aging of the sample material.

There is a wealth of information on phosphate precipitation *in vitro*[355, 365, 366, 376, 377, 377a]. Data indicate that morphology, composition and structure of the precipitate is a function of a number of environmental parameters most notably temperature, pH, kind and concentration of electrolytes, and the presence of organic matter. It is of significance that slight modifications in the experimental parameters have pronounced effects on the final product[370]. Most relevant is a recent study of precipitates of calcium phosphates formed in the physiological pH region[365, 366], and at temperatures of 25 and 37 °C. The first precipitate is ACP which – depending on molar Ca concentration and pH – readily transforms into octacalcium phosphate or brushite. The metastable crystallites are subsequently converted into calcium-deficient apatite. It has been proposed that ACP represent an agglomeration of hydrated calcium and phosphate ions with little or no three dimensional order[378]. Loss of excess hydration water and structural ordering may lead to epitaxial outgrowth of metastable and stable crystalline phases. Thus, ACP may be a suitable template for nucleation of a secondary phase.

From above experiments we can infer that ACP can readily form under physiological conditions. However, the problem, as far as biophosphates are concerned, lies in the conversion of ACP into crystalline phases in a matter of a few hours. With this in mind it is hard to understand why bones should have such a high ACP content. Thus, the concluding statement is at best tentative:

ACP-formation, most likely mediated by some organic substrate, is the initial solid phase. The process is similar to the deposition of amorphous silica ($SiO_2 \cdot nH_2O$) in diatoms, radiolaria, siliceous sponges and silico-flagellates (see p. 81). The two amorphous compounds differ in that metastable ACP readily transforms into a stable crystalline substance either directly or via metastable crystalline prescursors such as brushite or octacalcium phosphate. Amorphous silica, on the other hand, is metastable because the conversion into crystalline phases such as quartz, tridymite or cristobalite is kinetically slow at physiological temperatures. Consequently no crystalline SiO_2 phases are produced during the lifetime of an organism. Crystallisation proceeds only in the geological environment since thousands of years are needed to alter amorphous silica into quartz[379]. The final product is chert which is principally composed of cryptocrystalline quartz and some amorphous SiO_2 phases in various stages of hydration.

At the onset of biomineralization the mechanism of phosphate and silica deposition is essentially the same. Both start with a highly hydrated amorphous phase having glass-like physical-chemical properties. The kinetics of crystallisation of the two differs. ACP will rapidly alter in the direction of apatite in hours or days, whereas amorphous silica requires thousands of years or higher temperatures to yield quartz. As a result biophosphates may contain a variety of amorphous and crystalline phases, whereas biogenic silica is solely amorphous in nature.

(ii) Chemical Composition

During the deposition of biophosphates a selective fractionation of chemical elements occurs. Although this process has been known in principle for some time[380, 381], detailed studies had to await the development of new analytical tools such as the electron microprobe[382]. In order to relate the enrichment or depletion of elements in the course of biomineralization with time, precise time markes are needed[380, 383–389]. So the problem of elemental fractionation becomes a two-fold one, *i.e.* quantitative measurements a few micrometer apart and accurate timing at about hourly intervals.

In a recent investigation[382] the problem was overcome by employing the electron microbe for element analysis and using tetracycline labelling as a time marker. The objective of the study was to follow the fractionation of calcium, phosphorus, magnesium, sulfur and some minor elements as a function of time during periosteal bone formation in rat tibial diaphysis beginning in osteoid and continuing until steady state conditions in the mature bone are reached. The data are depicted in Figs. 41 and 42. Phosphorus and calcium sharply rise in the region of the mineralizing front and the curves closely resemble first order reaction curves. Half-times for calcium and phosphorus deposition are 0.49 and 0.36 days, respectively. The difference in the behavior of phosphorus and calcium comes best to light in the Ca/P curve (Fig. 41). At the mineralizing front and extending up to the 12% Ca level, a value of 1.35 is recorded. From there on, values steadily rise to approach 1.60 at maximum mineralization; plateau level is reached in less than four days. The general shape of the magnesium curve is similar to the calcium curve, except that the enrichment factor is lower by at least an order of magnitude and the plateau level is reached sooner. In the case of sulfur the concentration drops sharply at the mineralizing front.

Other elements have been suggested to be involved in phosphate deposition, most notably silicon[390]. From a structural point of view this makes sense because SiO_4 and PO_4 tetrahedra are isostructural, and individual tetrahedra units are stacked together, giving rise to isotype crystal structures[48, 391–393]. There is some indication that this principle is operative in biosystems. X-ray diffractions analysis of inorganic substances in atherosclerotic aorta tissue reveals that the plaque powder contains phosphates, silicates and aluminates[1]. This relationship suggests that silicon may proxy for phosphorus under certain biochemical circumstances or yield silicates. Some complexed heavy metals are also mentioned to induce mineralization[394], however, *in vivo* studies do not substantiate this inference[382]. For sodium, an element

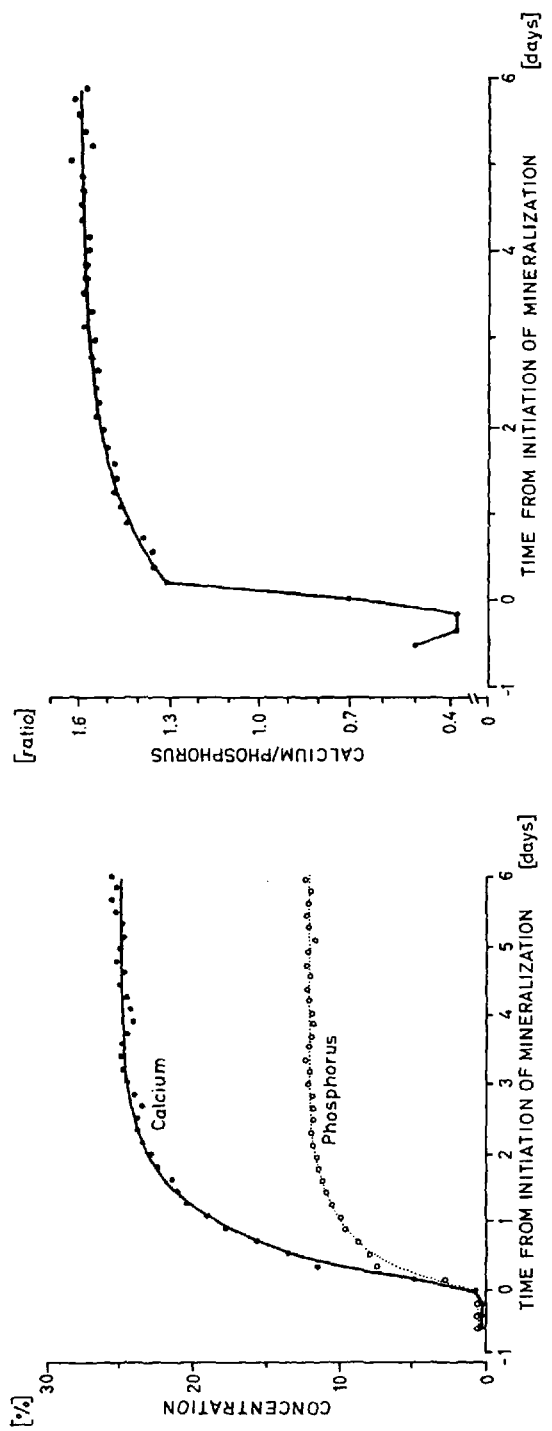


Fig. 41. Calcium and phosphorus concentrations and molar Ca/P during mineralization in normal rats. Each point is a mean value obtained from individual analyses in eight rats (after Wergedal and Baylink[382])

quite abundant in biophosphates, only a few systematic measurements at micrometer intervals have been undertaken[407]. In light of the crystallography of biophosphates, the observed elemental fractionation during mineralization may be interpreted as follows:

Mineralization proceeds in three steps, first, amorphous calcium phosphates and/or brushite are laid down causing a shift in Ca/P from 0.4 (osteoid) to about 1. Secondly, subsequent formation of octacalcium phosphate or Ca-deficient apatite causes a shift in Ca/P to 1.35 and the ratio remains almost constant until a calcium concentration of 12% is reached (= 50% mineralization); this state lasts for a few hours or perhaps days depending on the environmental circumstances. The final step is characterized by a gradual adjustment of Ca/P towards 1.60 and an exponentially decreasing deposition rate, reaching zero growth at a Ca-concentration of 25%. During this phase, the metastable mineral matter converts to apatite which mineralogically is a solid solution between the two endmembers hydroxyapatite and dahllite.

A topic of considerable controversy is the question of carbonate incorporation in the apatite lattice since carbonate apatite does not precipitate from aqueous solutions[28, 395–398]. Carbonate apatites (phosphorites) forming in marine environments[134] are considered metasomatic alteration products of calcium carbonate, and as a result carbonate content decreases and phosphate content increases with time[399]. In biophosphates, the situation appears to be just reversed in that carbonate content increases with bone maturation and it was argued that "the similarity between bone mineral and naturally occuring CO_3-apatite ends before it begins"[397].

Deposition of mineral matter is limited by diffusion of calcium and/or phosphorus to the site of deposition[400]. Since the transfer of both compounds is enzymatically controlled (see p. 21) equilibrium relationships may change environmental settings critical for the deposition of minerals. For instance, by limiting carbonic anhydrase activity in one direction, a pool of H_2CO_3 may build up at the site of deposition; in the reverse situation HCO_3^- will concentrate. As a consequence, $Ca(HCO_3)_2$ or $CaCO_3$ will be generated which can readily convert to a carbonate phosphate along the reaction scheme previously proposed[399]. However, it is conceivable that a true carbonate phase ($CaCO_3$) can have a temporary stability, thus accounting for the presence of calcium carbonate in medullary bone of laying hens[401, 402] which could be preferentially utilized in the formation of eggshell.

A number of ions are known to inhibit apatite nucleation and crystal growth. This has been established by *in vitro* experiments[403, 404] and calciphylaxis[38]. One strongly inhibiting ion is magnesium. The shape of the magnesium curve (Fig. 42) relative to that of calcium is thus quite revealing because magnesium reaches its plateau value of 0.4% at a calcium concentration of only 15%. This phenomenon may imply that Mg^{2+} ions act only during the first phase of mineralization possibly as an inhibitor in apatite formation; another possibility is that Mg^{2+} is discriminated against in the apatite lattice. Other ions, such as Pb^{2+} or Fe^{3+} were found to induce phosphate nucleation[405]. For details see Refs.[38, 404].

The drop in sulfur content at the mineralizing front is best explained by a loss of mucopolysaccharides prior to or at the onset of phosphate deposition[406].

In summary, crystallographical and chemical data support the inference that deposition of biophosphates is a multiple step process. In the initial phase, ACP's

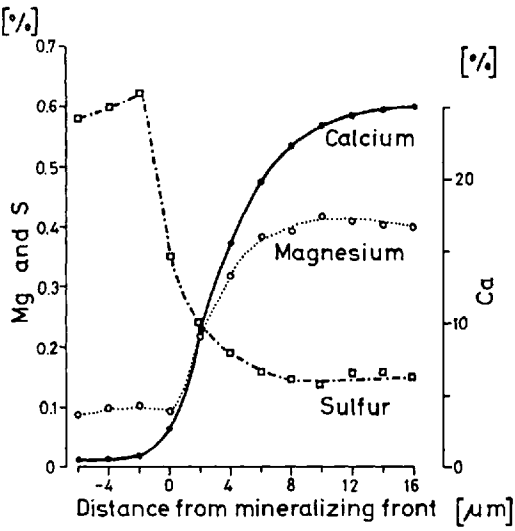


Fig. 42. Calcium, magnesium and sulfur concentrations during mineralization in normal rats. Each point is a mean value from 10 analyses (after Wergedal and Baylink[382])

and/or brushite are the principle deposits and mineralization rate is nucleation controlled. The material readily converts into octacalcium phosphate which in due time will yield Ca-depleted protoapatite or apatite. Carbonate content in phosphates increases as a function of exponential slow down in mineralization (= maturation of material). Rate controlling factors in crystal growth and conversion include: temperature, presence or absence of mineral inducing or inhibiting agents, diffusion parameters, and the species-specific physiological settings.

(iii) Organic Matrix

Specialized vesicles or cells generate a microenvironment for concentration of calcium and phosphate, and synthesis of organic molecules critical in mineralization. Enamel, dentin and bone formation is initiated by mesodermal cells known as ameloblasts, odontoblasts, and osteoblasts respectively. Similar cellular structures exist for the development of other hard tissues. Of the many organic compounds required for oriented mineral growth, only a few finally end-up as organic matrix in biophosphates; the rest is somehow "lost" in the course of mineralization. The amount of organic matrix varies considerably among biophosphates; bone and dentin have values around 25 per cent (dry weight), whereas in mature enamel the organic matter content is only about 1 per cent[14, 408–411].

In contrast to calcareous invertebrates with their species specific shell organic matrix[213–221], apatite depositors follow a rather conservative pattern set by collagen for bone, scale, and dentin deposition, or by a keratin-type protein in the case of enamel formation[412]. Nevertheless, a certain spread in the concentration of amino acids can be recognized if one, for instance, compares dentin of species en-

Table 7. Geometric means of amino acid in mineralized and unmineralized tissues (in residues/1000)[225]

	Dentin				Unmineralized Tissues				
	Elasmo-branchii	Pisces	Reptilia	Mammalia	Collagen	Keratin	Fibrinogen	Elastin	Resilin
[1]	(9)	(4)	(7)	(10)	(5)	(5)	(5)	(3)	(3)
OH-PRO	65	72	82	94	82			14	
ASP	84	75	60	62	49	65	33	9	101
THR	21	27	23	20	21	58	15	17	31
SER	50	55	50	41	44	103	131	13	80
GLU	60	80	84	81	74	111	18	21	48
PRO	101	104	123	124	120	75	7	96	79
GLY	354	330	325	324	325	84	292	332	383
ALA	112	106	109	105	113	54	281	236	107
CYS (half)	2	0.3	0.3	0.3		114			
VAL	27	20	18	20	20	68	24	126	28
MET	3	4	1	1	6	0.1		2	
I-LEU	20	10	12	9	12	34	12	19	17
LEU	25	24	24	27	26	74	13	53	23
TYR	1	2	1	2	3	25	46	17	27
PHE	5	8	10	9	14	26	5	31	26
OH-LYS	5	8	5	7	7				
LYS	14	18	17	17	25	23	7	7	6
HIS	3	5	2	2	5	6	3	2	9
ARG	35	40	49	45	48	59	24	7	35

[1] Number of analyses.

Table 8. Composition of bovine cartilage and bone[413, 414]

	Ash[1])	Coll.[2])	CS	KS	SA	OP	Total organic
	% of dry, mineral free weight of tissue						
Calf							
Articular	6.2	63.7	25.3	3.69	0.52	9.6	102.8
Epiphyseal	25.2	56.2	33.9	4.36	0.69	14.7	109.9
1 ° Spongiosa	69.7	75.8	1.9	1.29	0.41	10.6	90.0
2 ° Spongiosa	69.3	79.5	1.1	0.70	0.35	6.6	88.3
Diaphyseal	70.6	79.2	0.8	0.86	0.25	4.7	85.9
Fetus							
Articular	9.6	46.1	35.6	4.57	0.87	22.0	109.2
Epiphyseal	11.3	38.2	36.9	3.12	0.86	26.9	106.0
1 ° Spongiosa	72.3	71.2	2.6	2.69	0.65	11.8	89.0
2 ° Spongiosa	71.3	72.6	1.6	1.75	0.48	12.8	89.3
Diaphyseal	73.6	96.3	1.7	1.51	0.54	7.1	107.1

[1]) Ash in total dry tissue.
[2]) Coll. = collagen; CS = chondroitin sulfate; KS = keratan sulfate (calculated from glucosamine values); SA = sialic acid; OP = other protein, calculated from total nitrogen − (collagen + hexosamine nitrogen).

Table 9. Amino acid composition of human bone, dentine and enamel (residues per 1000)

	Human bone	Human dentin	Human enamel (developing)	Human enamel (mature)
OH-Proline	100	101		<8
Aspartic acid	47	55	30	54
Threonine	18	19	38	42
Serine	36	38	63	119
Glutamic acid	72	73	142	106
Proline	123	115	251	137
Glycine	319	319	65	193
Alanine	114	112	20	53
Cystine (half)			<4	4
Valine	24	25	40	32
Methionine	5	5	42	34
Isoleucine	13	10	33	19
Leucine	26	26	91	66
Tyrosine	5	2	53	23
Phenylalanine	14	14	23	33
OH-Lysine	4	8		4
Lysine	28	23	18	26
Histidine	6	5	65	19
Arginine	47	47	23	28

compassing a wide phylogenetic range (Table 7)[225]. Similar differences exist when comparing bones or enamels from various vertebrates. Such slight variations are probably due to admixtures of other proteinacous substances to the main collagen or keratin phases. As an example, the composition of calf and fetal long bones is presented (Table 8), showing trends in the distribution of proteins and mucosubstances from the cartilage towards the bone-cartilage interface[413–415]. Glycosaminoglycan, keratan sulfate and sialoprotein coexist with collagen. The data show that the majority of noncollagenous material in the bone matrix are glycoproteins, whereas glycosaminoglycans form the major noncollagenous component of epiphyseal cartilage. Bone sialoprotein and the sialo-glycoprotein of dentin have related chemical and physical properties[416]. Other organic compounds identified in bone and dentin include lipids and phospholipids[417–419], and albumin[420].

During enamel maturation, the protein content rapidly falls[14, 409–410], and fractions rich in proline and histidine are preferentially lost[421–424]. Newly formed enamel has the histochemical characteristics of a keratin[425] and it resembles in amino acid composition soft keratin of epidermis. It is interesting to note that calcified keratins – feathers, hairs, horns, nails, baleen – are quite common[412]. The small quantity of protein which eventually remains in mature enamel resembles one of a group of ectodermal globular proteins[425]. The idea has been advanced that during maturation enamel proteins are in a continual state of flux[408]. Representative amino acid analyses of developing and mature enamel, dentin and bone are depicted in Table 9.

A number of invertebrates deposit phosphate in their shell structures[24]. In the articulate brachiopod shell – *Lingula* – apatite crystallites occur up to about 1000 Å long. Little is known, however, on the nature of shell organic matter, except that the amount of chitinous material exceeds that of protein fraction by a factor of two[408].

(iv) Organic-Inorganic Interactions

In almost all instances of biological mineralization fibrous proteins represent the bulk of the organic matrix. In the past, this phenomenon has been interpreted to mean that proteins such as collagen, keratin or elastin are the key elements in mineralization by providing nucleation sites and at the same time offering structure and space for oriented crystal growth. However, with the advance in the field of biomineralization this model came under severe attack. At present, there is no universal concept which could explain all the intriguing facets of phosphate deposition in cellular systems.

Reconstituted acid-soluble collagen from various mineralized and unmineralized tissues have been examined for their potential to pick up calcium and phosphate from buffered solutions, and for their capacity to induce nucleation of a mineral phase[426–434]. Some collagens were good, others poor catalysts[428, 429] and apatite deposition proceeded in the presence of soft as well as of hard tissue collagens. The uptake of calcium ions requires the presence of phosphate ions and *vice versa;* the Ca/P ratio is close to that of apatite (1.5–1.8)[431]. Study of exchange reactions by isotope tracers between solvent und substrate revealed that in absence of either

calcium or phosphate in solution no exchange with the matrix bound calcium and phosphate takes place; presence of ion and counter ion is required[427]. Potent inhibitors of uptake include methylendisphosphonic acid and its derivatives which are also known to inhibit calcification processes in the living organism. Mg^{+2} and F^- ions inhibit net calcification but not exchange reactions. These findings suggest that reactions are not due to the reversibility of the net calcification reaction but represent an independent equilibration of mobile ions; site specific inhibitors are indicated.

Several lines of evidence indicate that calcium and phosphate are bound to the matrix in the form of apatite. The material has been examined under the electron microscope and by X-ray diffraction technique[426, 435, 436]. Most significant is the fact that the crystallites accentuate the 640 Å spacings of collagen[435]. As a general rule, beginning calcification is characterized by the development of parallel rows of single apatite crystals which are aligned with their crystallographical *c*-axis in the direction of the fibril axis of collagen[436].

Striking similarities in amino acid composition exist between elastin and collagen[437] (Table 7) and both exhibit a high affinity for calcium ions. It is, therefore, of interest to compare the binding capacity of calcium and phosphate to purified elastin. Data show[438] that the amount of bound calcium is dependent on pH and Ca^{2+} concentration and that no binding of phosphate is observed at physiological pH. Carboxyl and amino groups were assumed to be the calcium binding sites. In a related experiment[439] sulfhydryl groups were suggested as the nucleating species in calcified elastin. In substituting pure aqueous solutions for methanol-H_2O mixtures and testing the uptake of calcium by elastin, an almost pH-independent relationship was found[440]. Also, ionic strength did not effect the uptake characteristics of calcium. Binding of phosphate to elastin was not observed in either the presence or the absence of methanol.

How can these controversial experimental findings in the collagen and elastin systems be reasonably explained? In the first place, lack of phosphate binding to elastin can best be accounted for by the absence of Ca^{2+} in the NaH_2PO_4-containing solvent media (counter ion – effect). The experiments, however, indicate that binding of calcium to the matrix must procede that of phosphate or other counter ions. Addition of methanol to the solution will elicit conformational changes in elastin; completely aqueous medium favors less ordered conformation. As a result highly ordered elastin binds calcium largely independent of changes in hydrogen ion concentration and ionic strength, while in less ordered elastin calcium fixation is strongly controlled by pH and salinity. These findings may imply that in elastin and possibly in collagen too neutral sites exist for calcium ion binding[440–442].

The collagen molecule is comprised of three polypeptide subunits (α-chains) giving rise to a triple stranded helix[31,36, 443–447]. Biosynthesis involves formation of procollagen which is characterized by peptide extensions termed "registration peptides" that serve to bring the three α-chains rapidly into its triple helix conformation[448, 449]. Procollagen is a disulfide-stabilized molecule of molecular weight of about 360.000 daltons[450–452]. Interchain disulfide bonding seems to control whether the pro-α-chains are in a triple helical conformation or in random coil conformation. It is of significance that the rate of procollagen synthesis varies among

different types of cells. While disulfide bonds are needed to speed up the triple helix formation, hydroxyproline increases the thermal stability of the collagen triple helix and thus maintains triple-helical conformation in cellular systems[453–455].

The elastin molecule is usually regarded as a gel of randomly coiled peptide chains, cross linked to each other. A different elastin model – "oiled coil" structure – has recently been proposed[437] which provides a molecular basis for the behavior of the protein. The main feature of the model is a broad coil in which glycines occupy the exterior positions exposed to solvent, while proline, valine and other hydrophobic residues are buried; most of the peptide backbone is hydrated. Soluble elastin has a chain length of about 840 residues, based on a molecular weight of 70.000 daltons[456]. Chief differences between soluble and mature elastin are the loss of lysine and formation of cross links during maturation.

Collagen and elastin have a high glycine content, and exhibit high affinities for calcium ions; glycine favors the formation of β-turns and associated conformations of the type developed in peptide antibiotics[457–460]. These antibiotics – for instance valinomycin, enniatins, gramicidin – all utilize acyl oxygens for metal ion coordination. The enniatin cation complex[460] showing the cation to be held centrally by an octahedral field of six acyl oxygens, is depicted in Fig. 43. Similar structures are envisioned for elastin and collagen[440–442], in that the calcium is bound through uncharged coordination groups, *i.e.* neutral sites in the protein matrix. Consequently local areas on the fibrous proteins will become positively charged which in turn will attract charge-neutralizing ions such as phosphate and carbonate. Once a given site is neutralized, adjacent loci can bind calcium ions. The driving force for calcification is the affinity of calcium ions for the neutral binding site. In the case of collagen with its somewhat higher content in carboxyl and hydroxyl functions, oxygens other than acyl oxygens may be involved in Ca^{2+} coordination polyhedron, a situation comparable to $Ca^{2+}O_6$ octahedra conformations in the EF hands of troponin[202, 203a] (Fig. 21).

One of the key arguments for neutral site binding is the presence of β-turns and associated conformations. This puts certain restrains on the structure of the fibrous protein. For elastin, conformations with bound calcium are likely to be inside-out with respect to hydrophobicity. Such structures are acceptable only for molecules functioning in a non-polar environment (cell-membranes) but not for a hydrated elastin fibre. Binding of calcium would stabilize a rigid inside-out conformation[437].

In addition to fibrous proteins, protein-carbohydrates (muco-substances) represent a substantial portion of the organic matrix in biophosphates (*e.g.* Table 8). They have been linked in one way or another with mineralization[461–469]. On structural grounds, bone and dentin sialoproteins and glycosaminglycan could provide sites for the coordination of calcium ions; diagrams illustrating a possible structure for the carbohydrate moiety of these mucosubstances are given in Fig. 44. Carbohydrate groupings are covalently bound to a protein, and many sites exist for metal ion coordination.

Lipids and phospholipids too accumulate at the site of active mineralization. It has been proposed that phosphatidylserine can combine with hydrated metal ions and form a bi-or possibly a tridentate complex[417]. In forming such a complex, the hydrophilic properties of Ca^{2+} are decreased and ligands could associate with the

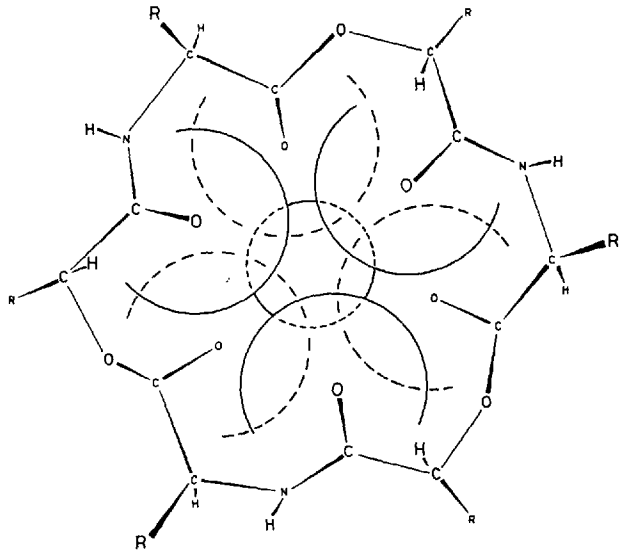


Fig. 43. The enniatin-cation complex, showing the cation to be held centrally by an octahedral field of six acyl oxygens (after Ovchinnikov *et al.*[460])

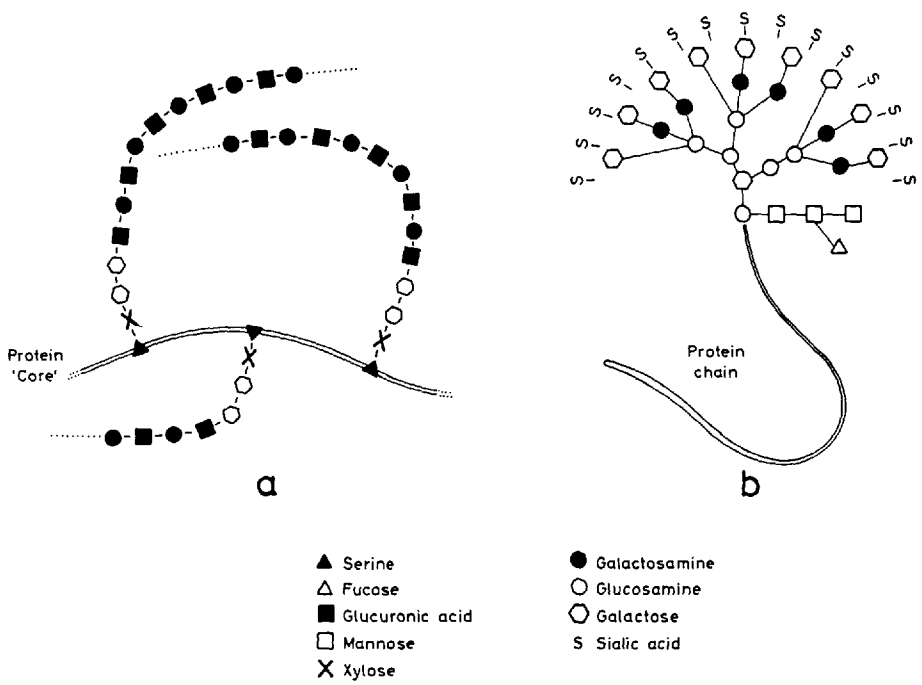


Fig. 44. Diagram illustrating the structure of a section of (a) protein-polysaccharide molecule, and (b) bone sialoprotein (Herring[414])

calcium-lipid chelate. Thus, this group of compounds may be active in the phosphorus and calcium transport systems of mineralizing tissue.

In conclusions, many schemes have been developed for metal ion – phosphate – organic matter interactions in biomineralization. A variety of organic compounds of the kind present in mineralized tissues were found to coordinate calcium ions at neutral or functional sites; and in many instances metal ion coordination was accompanied by the binding of phosphate. Although a wealth of information exists on the organic-inorganic interplay, data could not be reduced to a point where a simple model on biological mineralization could be formulated.

(v) Calcium Regulation

Concentration of calcium ions in the cytoplasm is very critical to cell function. It has been proposed that mitochondria play a significant role in regulating the concentration of free Ca^{2+} in the cytoplasm by their very high affinity for Ca^{2+} and their capacity to segregate Ca^{2+} very rapidly and, apparently, reversibly[470–476]. Since mineralization is under strict cellular control, the idea has been advanced that the mitochondria are responsible for concentrating the essential ions of the crystalline phases. The existence of an energy dependent calcium transport in mitochondria is well documented. A number of criteria indicate that transport of Ca^{2+} is a carrier-mediated process. In addition strict correlation has been found between high affinity binding and energy-driven transport in mitochondria. During massive loading of Ca^{2+} it was established that Ca^{2+} uptake requires electron transport and that up to 2.6 μmoles Ca^{2+} per mg mitochondrial protein could accumulate; maximal loading requires only a few minutes. In mitochondria from vertebrate species the number of Ca^{2+} ions required to activate each energy conserving site ($Ca^{2+}/\sim$activation ratio) is about 2. Ca^{2+} ion addition causes H^+ ion ejection in a one to one ratio. The manner in which one-half of the electrical charge of the entering Ca^{2+} is compensated is not known yet.

As to the nature of the Ca^{2+}-carrier one view is that the electron-carrier molecules *per se* function directly as the Ca^{2+}-carrier system. Alternately, a molecular system independant of the electron carrier of the respiratory chain, but responsive to electrochemical gradients generated by electron transport acts as calcium carrier. Some recent findings support the latter view in that calcium transport as well as high affinity calcium binding is possibly linked to a Ca^{2+}-specific glycoprotein. The glycoprotein is absent in mitochondria which are devoid of both high affinity calcium binding and active calcium transport. The calcium binding glycoprotein is located at the inner and outer mitochondrial membrane[477]. This spatial arrangement may confer specificity for the transportation and binding of calcium by mitochondria. Under the electron microscope mitochondria can be seen with large numbers of electron-dense granules in which calcium and phosphate ions are concentrated in a stoichiometric ratio of 1.67[475]. Consequently, for each pair of electrons traversing each energy-conserving site, 1.67 molecules of Ca^{2+} and one molecule of phosphate are accumulated during massive loading. There are claims that needle-shaped crystals, probably apatite, are present in some Ca^{2+}-loaded mitochondria[478, 479].

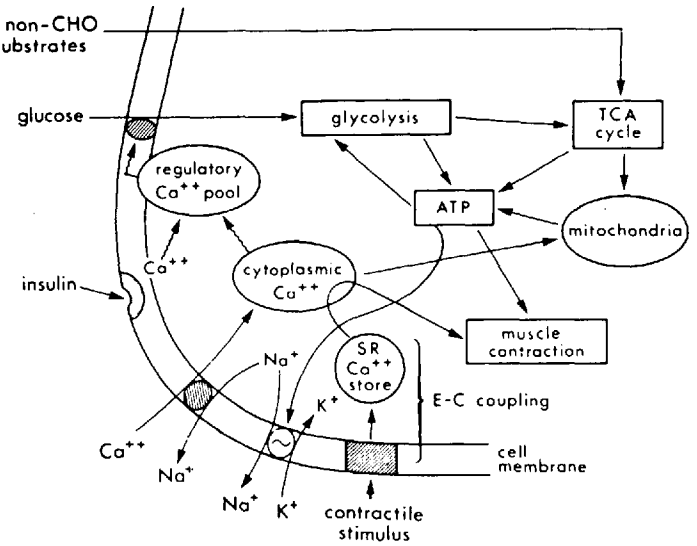


Fig. 45. Interactions between glucose transport and modulating factors. Full arrows indicate metabolic pathways and ion fluxes; wavy arrows show proposed modulating effects via a hypothetical regulatory Ca^{2+} pool (Elbrink and Bihler[483])

Others suggest that these needles are analytical artifacts and that *in vivo* the clusters are amorphous[476].

Recent work on osteoblasts and osteoclasts has shown that osteoblasts in the region of new osteoid have a higher mitochondrial granule number than do the adjacent flat osteoblasts associated with more mature bone[480]. Osteoclasts show an intracellular mitochondrial gradient: The mitochondria adjacent to the resorbing surface are filled with granules while those on the vascular surface are free of granules.

These data may imply that cellular mechanisms for calcium transfer and homeostasis are intimately tied up with mitochondria and that biomineralization is an essential element in Ca^{2+}-regulation processes[481, 482]. For a review on the role of mitochondria in the deposition of amorphous calcium phosphate in the early steps of biological calcification, see[473, 474].

The metabolic pathways, ion fluxes, and glucose modulating effects via a hypothetical regulatory Ca^{2+} pool are depicted in Fig. 45[483]. It is probable that binding of Ca^{2+} to specific sites at the internal face of membranes alters conformation of some membrane constituents, thus allowing transfer or passage of hydrophilic substances across the lipid barrier. Membrane transport of a sugar carrier such as glucose is thus mediated by means of change of membrane permeability. This transport is characterized by Michaelis-Menten saturation kinetics, chemical specificity and restructuring of substrate and membrane components rather than by simple diffusion processes. Participation of calcium ions in the modulation of sugar transport is in line with data on the interference of sugar transport across membranes by certain ions antagonistic to calcium[485]. The cytoplasmic Ca^{2+} level is affected by $Ca^{2+}-Na^{+}$

exchange and by excitation-concentration coupling in the sarcoplasmic reticulum system. Furthermore, rapid mitochondrial oxidation may deplete the regulatory Ca^{2+} pool. In turn, the regulatory Ca^{2+} pool will recharge by means of a feedback system from storage sites inside the cell or through some form of energy-dependent "ion pump" system. Such an elaborate apparatus requires a valve system for the fast removal of calcium ions in order to deal with excess calcium ions entering the organism from the environment. Biomineralization could serve as such an excretionary system. It has principally been invented not for storage of calcium, phosphate or carbonate but for the efficient removal of surplus calcium from within the cell.

(vi) Mineralization: Ca^{2+} Coordination

In 1962 it was said that "the nature of the local mechanism of calcification is one of the most important unsolved problems in biochemistry"[484]. This statement still holds true in spite of all the significant work that has been done in the years in between. There is no easy way out for a reviewer who tries to coordinate the various findings, hypotheses, and ideas that have been made in this direction but no universally acceptable model on the mechanisms of biomineralization has yet emerged. There is no need to duplicate the efforts of other reviewers. Instead, the present work will only concentrate on one key aspect of biomineralization where some new insight has recently been gained: binding and transfer of calcium ions, and subsequent formation of mineral phases.

We have seen that Ca^{2+} ions play a key role in cellular systems. In first place, they are involved in energy-linked activities and in second place they can bind to specific sites and introduce conformational changes. For instance, Ca^{2+}-binding to membranes results in a conformational rearrangement of the repeating units which in turn alters the permeability characteristics of the inner membrane. Or let us take the case of muscle activities; Ca^{2+} will provide the "correct" conformational settings between molecules participating in excitation-contraction coupling. Therefore, one might expect that Ca^{2+} ions follow a similar pattern in the course of mineralization.

One of the most significant findings, in recent years in the field of biomineralization is the identification of calcium-accumulating vesicles in intercellular matrices of hard tissues[485, 486]. The size of the matrix vesicles is about 100 nm in diameter. A trilaminar membrane envelopes the vesicle which appears to be derived from plasma membranes. This relationship suggest that – in a wider sense – matrix vesicles are Golgi-elaborated structures. In a closer sense, they are linked to calcifying cells such as chondrocytes from which they became separated by budding; their *de novo* formation in the cell matrix can be ruled out. Isolated matrix vesicles are rich in lipids and phospholipids which show a high affinity for Ca^{2+} [486–490]; at the same time, the vesicles are depositories of inorganic pyrophosphatase, ATPase and alkaline phosphatase[485]. Lack of acid phosphatase activity, β-glucuronidase and cathepsin D indicates that we are not dealing with lysosomal structures. The high content of phospholipids, and here of phosphatidylserine and phosphatidylinositol, cholesterol and glycolipid in almost twice the cellular amount represents a clue to the origin of the vesicles and the mode of Ca^{2+} fixation.

Metal ions and cholesterol are capable of reducing the volumes of chain molecules and of initiating contraction of a monomolecular film to a condensed state[491]. For lipid membranes this will result in formation of rigid membrane layers[48]. Phase transformation represents the most remarkable aspect of this coacervation mechanism. With respect to matrix vesicle formation, the coordination state of metal ions, the type of metal ion, and the amount and positioning of cholesterol within the membrane of a developing calcifying cell may locally change. This will introduce contraction or expansion of the ionic boundary layers in membranes (see Figs. 4 and 5) and result in budding and formation of isolated vesicles. Such a development has interesting biochemical consequences, since not only a physical but also a chemical separation is achieved.

In the process of Ca^{2+} -fixation to the internal face of the vesicular membrane, it is conceivable that mucosubstances act as Ca^{2+} carrier and transfer their calcium to the membrane surface. Phosphatidylserine and other phospholipids are potent acceptors of this calcium. Since Ca^{2+} has coordination numbers between 6 and 9, various kinds of polyhedra networks can arise. Phosphate-cation chains develop:

$$= PO_4 = Ca = PO_4 = Ca = PO_4 =$$

that become cross-linked into a planar pattern. An especially interesting structure is one of corrugated layers of calcium monohydrogen phosphate, $CaHPO_4 \cdot 2\ H_2O$[64], which has been proposed as the initial mineral phase in biophosphates[492].

Following the separation of the vesicles from their stem cell, the process of calcification continues implying that Ca^{2+} is actively moving into the vesicle. In mitochondria and sarcoplasmic reticulum membranes we have followed in some detail the movement of Ca^{2+} in and out of the membraneous systems. The abudance of certain enzymes inside the vesicle supports the inference that a similar acting system is at work in matrix vesicles. Presence of pyrophosphatase at the vesicle membrane is needed to hydrolyze inorganic pyrophosphate (PP_i) into phosphate ($2P_i$). Pyrophosphate is a known inhibitor of mineralization[487,493–496]. Intravesicular calcification terminates with the rupture of the vesicle and the release of mineral matter and vesicle content at or close to the mineralizing front. The idea has been advanced that accumulation and growth of intravesicular crystals causes the vesicles membrane to rupture[485]. However, in analogy to mitochondrial systems[407], it is conceivable that swelling and eventual burst of the vesicles is proceeded by passive equilibration of alkali metal salts present in the medium, and Ca^{2+} ions may potentiate this swelling.

It was stated that hydrated calcium monohydrogen phosphate in amorphous or cryptocrystalline form is a potential precursor in the formation of hydroxyapatite because the structural position of Ca^{2+} on (010) and (110) crystal planes of both minerals essentially correspond to one another[492]. These planes of calcium ions could easily serve as transition boundaries with little distortion of crystal structure; the same holds true for octacalcium phosphate or defect apatites. Thus apatite may form from amorphous or microcrystalline calcium monohydrogen phosphate possible via octacalcium phosphate or defect apatites. This process may already start inside the matrix vesicles and continue during extravesicular activities.

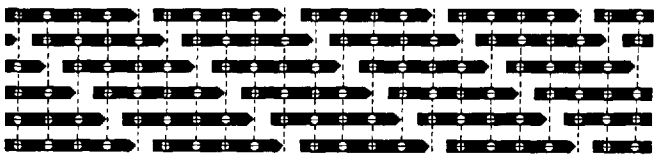


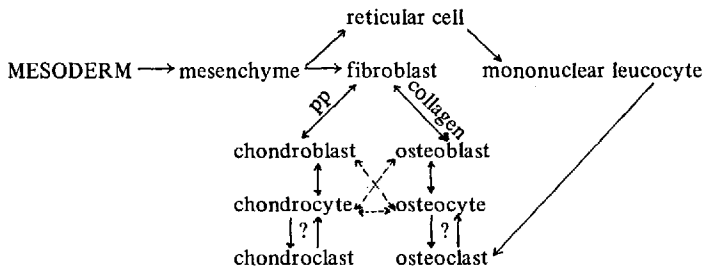
Fig. 46. Molecular overlap and hole zone in collagen fibrils (after Höhling[500] and Münzenberg[354])

Following the rupture of the matrix vesicle, the phospholipid template plus mineral matter interacts with an organic substrate which is principally collagen in the case of dentin, cartilage and bone, and some keratin-type protein in the case of enamel. The ability of collagen to form biophosphates appears to be linked to the existence of "hole zones" which develop between the ends of collagen molecules (Fig. 46). Collagen fibrils and the rather lengthy needles of apatite crystals lie paralel to one another[498–500]. This alignment merely reflects the shape of the space available to a developing apatite crystal. It is unknown whether the collagen binds the essential elements in the form of single ions, ion clusters, amorphous phosphates, or microcrystalline material. It is also not clear yet, whether this binding is specific or non-specific. The issue is furthermore complicated that some collagens mineralize *in vitro* and *in vivo*, while others do not. In view of the data on matrix vesicles which indicate formation of crystalline matter during the vesicular stage of mineralization it is proposed that these minerals serve as crystal seeds which – when placed in hole zones – enlarge in size. Thus the alignment of collagen fibrils and apatite crystals as seen at late stages of mineralization not necessarily implies that collagen is the nucleator of apatite. It is more probable that much of the apatite in fully mineralized tissues was formed by mineral growth upon pre-existing crystals, whereby collagen excercises structural control. In this capacity, collagen has the same physiological function in phosphate deposition as the silk-type carrier protein in carbonate formation.

(vii) Cellular Differentiation

In an extensive series of experiments it could be demonstrated that bones, cartilage and fibrous tissues are produced from similar fibroblasts[501–503]. Furthermore, transformation of one skeletal tissue into another one in either direction is possible. Thus, the former concept on the fixity of cell types which says that once a cell had differentiated into myoblast, chondroblast or osteoblast it could only produce muscle fiber, cartilage or bone, respectively, had to be revised.

For instance, bone growth may develop by cartilage formation which increases in size by interstitial expansion and – following calcification – remodels to form bone; alternately, bone can form directly from connective tissue-like cells[504]. Such transformations commonly proceed at the cellular level, *e.g.* chondrocytes become osteoblasts and vice versa. A flow diagram illustrating the transformations of cells within skeletal tissues is given below[503]:



pp = protein-polysaccharide
⟵ differentiation
⟵---- metaplasia

The fact that cartilage, bone and fibrous tissue generating cells are readily interchangable indicates that the individual progenitor cells share the same genetic information. The question thus comes up what factor or cofactors determine the direction of cellular flow?

Factors which evocate osteogenesis and chondrogenesis have been enumerated and discussed at length[503, 506]. Most essential appears to be the degree of vascularity and the presence or absence of mechanical stress. For instance, compaction and high oxygen levels induce bone formation; compaction and low oxygen levels yield cartilage; and tension and high oxygen levels cause fibrous tissue formation[501, 502]. These factors have been related to a possible common metabolic process involving collagen and chondroitin sulfate syntheses. Enhancement of collagen production favors osteogenesis whereas enhancement of chondroitin sulfate production favors chondrogenesis[508]. It appears that the ultimate control device is positioned in the Golgi area; alteration of the Golgi-cytoplasmic relation might change the proportions of excretionary or secretionary products[507]. For further details see Ref.[503].

(viii) Evolution of Biophosphates

The fossil record shows that the oldest eukaryotes – microscopic unicells – occured earlier that 1.300 million years ago and possibly earlier than 1.700 million years ago[508–511]. An advanced level of eukaryotic organization was reached in about Bitter Springs time which dates back about 900 million years[512, 513]. This time marks the "invention" of eukaryotic sexuality which has been suggested as a possible trigger mechanisms for a marked increase in diversity and evolutionary rate among late Precambrian eukaryotes[514]. It is of note that megascopic size was attained more or less concurrently by both plants and animals. Recent findings[514ab] cast doubt on the validity of the proposed evolution of eukaryotes. It was suggested that the Precambrian fossil record has been seriously misinterpreted because degradational features of certain prokaryotes were explained as evidence for the presence of euka-

ryotic microfossils. Thus, for the moment, we have only the Ediacaran faunas as the first true sign of multicellularity in the geological record.

Whatever the real time sequence of eukaryote evolution may be, origin and development of the nucleated cell has implications for the understanding of the evolution of O_2 in hydrosphere and atmosphere since even the few anaerobic eukaryotes that presently exist require molecular oxygen for synthesis of some essential biochemicals[472]. Thus all eukaryotic cells are basically aerobic and it is most likely that they evolved from an aerobic protoeukaryote stem cell.

Critical concentration of oxygen for metazoan oxidative metabolism is placed somewhere near 3 per cent of the present atmospheric level. At such low O_2 concentration there is little oxygen to spare for compounds that are energetically expensive to produce[515]. One energy-consuming reaction is the enzymatic hydroxylation of proline and lysine which is needed to convert protocollagen into collagen. To yield high strength structural collagen in larger quantities, organisms had to await increase in oxygen content in atmosphere and hydrosphere. Since collagen is "the tape and glue of metazoan world" its uninhibited production must be guaranteed by the environment to support evolution to higher forms of live[515].

In invertebrates, collagen is uncalcified, whereas in vertebrates it occurs in the form of soft and hard tissue. Remains of the earliest vertebrates – bony armour – are found in sediments of the Ordovician (ca. 500 million years). At about that time, conodonts appeared in the stratigraphic column; mineralogically they are carbonate apatite. Conodonts are small tooth-shaped fossils 0.2 to 3 mm in size whose origin is in doubt; they are most likely remains of some unknown chordata that became extinct in Triassic time[516].

Excellent reviews have been prepared on the evolution of bone, cartilage, dentin and aspidin which is the tissue of dermal armour (exoskeleton) in some ancient vertebrates[517–521]. Controversy exist whether bone and cartilage arose independently of one another, or – if they evolved from the same progenitor cell – which is the more primitive tissue. At present the weight of evidence suggests that the first mineralized tissue has been a spherulitic calcified cartilage which has been present in the dermal armour of the first vertebrates[522–524]. This would be in line with the observation that low oxygen levels yield cartilage, while bone formation requires high oxygen content[501–503]. With the advance of oxygen, collagen formation was favored and the primitive hard tissue aspidin came into existence; eventually it evolved to true bone. It is of note that already in the early stages of biophosphate mineralization there are many examples of transformations of cellular tissues. For instance, a study on arthrodires leads to the inference that during evolution dermal bone can sink in to become cartilagineous[525].

In summary, phosphate deposition in cellular systems can be linked to evolution of oxygen in the earth atmosphere and hydrosphere. Only at certain critical O_2 levels can the organic substrate for mineralization be synthesized. Mineralization began with the energetically least wasteful matrix, *i.e.* cartilage and gradually progressed – with the advance in oxygen content – to aspidin and eventually ordinary bone and dentin. In the final analysis, however, this development has been forced onto the evolving organisms by Ca^{2+} as a consequence of the increasing demand and turn-over of calcium ions in membrane activities.

IX. Silica Deposition

(i) Biochemistry

Silicon is, next to oxygen, the most abundant element in the earth's crust. It occurs, at least in trace quantities, in most plant and animal tissues[526, 527]; in a variety of organisms it is considered an essential element. Spectacular is the enrichment of silicon in mineralized tissues of unicellular organisms, *e.g.* diatoms and radiolaria, and a few metaozoa, *e.g.* sponges and gastropods[7, 8, 528]. The mineral phase is always amorphous silica in various stages of hydration $SiO_2 \cdot nH_2O$.

In a series of experiments on diatoms one could show that individual diatom populations require a certain level of dissolved silica (orthosilicic acid)[529–531] and that declining ambient silica concentrations in natural environments may influence the sequence of seasonal successions[512]. Silicoborates have also been mentioned as growth factors[533]. It is conceivable that other silica-depositing organisms have their specific requirement for silica concentration in the environment.

Data on the physiology of silicon is limited, except that in a number of species its presence is needed for normal growth[534]. Tracer studies with ^{71}germanium on some diatoms indicate that a silikatase might be involved in silicon metabolism[535]. It is of note that alkaline and acid phosphatase exhibit no silikatase activity. Divalent sulfur-containing compounds, such as sulfide, methionine, cysteine and glutathione have a positive effect on silica uptake[536]; also water temperature and silica concentration in the aqueous phase control the Si-cycle in cellular systems[529–531, 537].

There is reason to assume that a fraction of the cellular SiO_2 is associated with plasma proteins[538]. Some vacuoles have been mentioned[539] which could serve as a depository of SiO_2 reserves. The association of SiO_2 with Golgi vesicles however, is certainly related to silica excretionary processes[540]. The importance of silicon as factor of normal growth and development is illustrated in the following experiment[541]:

Day-old chicks fed on low-silicon diet for about 3 weeks showed a retarded growth and development when compared to chicks fed the same diet plus a silicon supplement. Difference in weight gain was about 50 per cent and the bone structure in the low-silicon group was severely retarded. At present we do not understand the physiological role of silicon. Structurally and chemically, silica and phosphate are closely related to one another. SiO_4 and PO_4 coordination tetrahedra are isostructural. They differ only in the presence of a π-bond in the PO_4 tetrahedron, which causes certain restrictions in the three-dimensional linkage of PO_4 units during polymerization and, in contrast to SiO_4, one of the oxygens cannot function directly as a P–O–P bridge, except in the form of a hydrogen bridge. It is proposed that the physiological functionality of silicon rests on its ability to coordinate four oxygens in the form of SiO_4 thus generating four equally spaced Si–O–Si bridges. Under certain circumstances such a steric arrangement might yield a structural rigidity or conformation advantageous to the organism. Silicon could possibly form stable coordination complexes with aromatic *vic*-diols[542]; tannins, DOPA, or polyphenols are potential ligands. Also hydroxyl-containing amino acids – serine and threonine – are likely candidates for Si^{4+} coordination. The primary structure of many proteins frequently reveal typical serine successions:

Ser–Ser; Ser–Ser–Ser; Ser–X–Ser; Ser–X–Y–Ser

and some proteolytic and esterase enzymes have serine at the active center and threonine next to the active site[166]. In atherosclerotic aorta tissue, however, this ability is used to the disadvantage of the organism since the formation of silicates can be initiated[1].

(ii) Organic Matrix

When a siliceous skeletal tissue is examined, one always finds an organic coating in the case of diatoms or an axial filament in the case of sponge spicules; other siliceous organisms have related organic structures. Knowledge on the chemical composition of these organic materials may allow insight into the mechanism of silicification since organic and inorganic phases are intimately associated with one another. However, this close relationship combined with the generally small size of silica frustules or sponge spicules makes identification of the organic matrix rather difficult.

The presence of common sugars and amino acids was recognized quite early; and the number of distinctive sulfated polysaccharides and uronic acids led to the conclusion that the organic casing of diatoms is heterogeneous and complex[543,544]. The axial filament in sponges has a substantial protein component[545, 546] but its precise relationship to the carbohydrate fraction and quantitative amino acid composition is still in doubt. The amino acid composition of an HF-residue of siliceous spicules should be viewed with caution because the material may represent a mixture of surface tissue and axial components[547]. Of significance is the observation[546] that C/Pt replica of HF-etched spicule cross sections show concentric rings with spacing as small as 0.2 to 0.3 micron, or about 100 rings per spicule. These may represent growth increments similar to the ones observed in fish otoliths. The question whether individual rings are produced by organic coatings a few Å thick or represent subtle differences in composition or physical structure of the spicopal remains open.

Some of the aforementioned analytical difficulties have largely been overcome in a recent study on a selected group of diatoms and siliceous sponges, and the results are briefly summarized[548]:

All diatoms were cultured and cell contents and cell walls were individually analyzed for their amino acid and sugar content (Tables 10 and 11). Comparison of paired analyses support the view that the cell walls are free of cytoplasmic contamination. Further evidence are electron micrographs on isolated cell wall fractions which reveal uncontaminated bi-layer membranes (Fig. 47). In all instances, cell walls and cell contents are distinguished by their amino acid composition, and a few general trends come to light. Glycine and hydroxyl-containing amino acids (serine plus threonine) are consistently enriched in the cell wall, while acidic, sulfur-containing and aromatic amino acids show a consistent depletion. Hydroxyproline is virtually absent indicating the lack of collagen. The same relationships are established for the one analysis in Table 10 of a siliceous sponge which would support the inference that diatoms and siliceous sponges share the same type of proteinaceous matrix in their silica deposits. Sugars of the cell-wall carbohydrates (Table 11) are quite variable

Table 10. Distribution of amino acids (residues/1000) in cell contents and cell walls of 6 species of diatoms from various habitats. Column a: distribution in cell contents; Column b: cell walls. Bottom of table emphasizes the difference (Column b – Column a) between cell-wall composition and cell contents for those amino acids which show consistent trends. The sponge spicules are from *Cliona celata*, and the sponge tissue is from an unidentified siliceous sponge. AA: amino acids; HA: hexosamines

Amino acids	*Navicula pelliculosa*		*Melosira nummuloides*		*Melosira granulata*		*Cyclotella stelligera*		*Cyclotella cryptica*		*Nitzschia brevirostris*		Siliceous sponge	
	a	b	a	b	a	b	a	b	a	b	a	b	Tissue	Spicule
Aspartic acid	116	105	n.d.	128	128	117	119	103	124	136	113	83	111	117
Threonine	66	64	n.d.	57	67	83	54	70	65	103	62	74	76	45
Serine	82	99	n.d.	127	108	228	98	252	95	235	65	84	57	163
Glutamic acid	143	100	n.d.	91	107	64	109	59	120	61	141	145	102	68
Proline	80	66	n.d.	75	69	9	57	65	73	12	48	85	78	47
Glycine	116	131	n.d.	123	77	113	110	195	112	210	109	114	99	134
Alanine	75	107	n.d.	106	84	80	98	60	47	49	106	96	91	114
Cystine (half)	3	1	n.d.	4	2	2	2	1	9	2	8	1	4	6
Valine	64	68	n.d.	60	53	73	62	41	65	31	64	75	70	55
Methionine	15	9	n.d.	19	7	2	20	1	20	1	26	4	20	6
Isoleucine	41	53	n.d.	45	47	61	50	36	50	15	49	37	63	31
Leucine	84	104	n.d.	75	103	78	92	54	89	31	87	73	74	68
Tyrosine	23	21	n.d.	26	26	8	29	6	30	14	27	13	32	27
Phenylalanine	46	45	n.d.	42	35	17	46	12	47	13	45	52	52	14
Lysine	32	18	n.d.	16	59	29	35	25	42	50	32	36	20	64
Histidine	2	1	n.d.	1	3	3	1	5	3	11	1	8	11	27
Arginine	12	8	n.d.	4	27	32	17	15	8	27	17	20	41	20
Total amino acids (mg/g)	232	280	n.d.	124	73.9	7.3	170	79.1	114	30.8	222	21.9	15.6	0.74
Hexosamines (mg/g)	4.27	1.48	n.d.	0.81	9.23	0.61	14.7	2.71	11.5	2.85	14.0	1.15	1.55	0.76
AA: HA ratio	54	189	n.d.	153	8	12	12	29	10	11	16	19	10	0.97
Serine + threonine	+15		n.d.		+136		+170		+178		+31		+75	
Glutamic acid + aspartic acid	−54		n.d.		− 54		− 66		− 47		−46		−28	
Glycine	+15		n.d.		+ 36		+ 85		+ 98		+ 5		+35	
Tyrosine + phenylalanine	− 3		n.d.		− 36		− 67		− 50		− 7		−43	
Methionine + cystine	− 8		n.d.		− 5		− 20		− 26		−29		−12	

Table 11. Distribution of sugars (M %) in cell contents and cell walls of diatom species. Notation as in Table 10

	Navicula pelliculosa		*Melosira nummuloides*		*Melosira granulata*		*Cyclotella stelligera*		*Cyclotella cryptica*		*Nitzschia brevirostris*	
	a	b	a	b	a	b	a	b	a	b	a	b
Rhamnose	5.0	3.8	2.0	1.8	4.4	1.2	0.6	0.0	3.0	7.4	1.6	2.4
Fucose	4.1	9.2	1.0	25.6	4.4	0.8	2.3	0.6	7.8	12.2	5.4	42.5
Ribose	0.9	0.0	1.8	0.0	1.4	0.0	0.8	0.0	4.9	0.0	2.5	4.7
Arabinose	0.0	0.0	0.5	0.3	0.3	0.6	0.3	2.0	0.0	0.0	0.0	1.7
Xylose	1.2	3.1	7.2	10.9	6.7	38.9	2.6	18.0	4.6	17.8	1.7	4.3
Mannose	20.4	48.5	12.4	56.8	13.7	6.5	4.1	13.6	9.2	37.2	19.3	20.2
Galactose	20.4	9.9	10.4	3.7	8.0	5.4	6.9	22.4	4.0	12.2	13.2	9.5
Glucose	48.0	25.9	65.1	0.9	61.2	46.7	82.4	43.3	66.4	13.0	56.4	14.6
Total Sugars (μ moles/g)	127	162	154	211	526	154	813	205	346	37.6	373	49.4
(mg/g)	22.7	28.7	27.1	36.8	93.1	26.0	144	35.8	68.0	6.48	66.5	8.36
AA/Sugar Ratio	10.2	9.8	n.d.	3.4	0.79	0.28	1.2	2.2	1.7	4.7	3.3	2.6

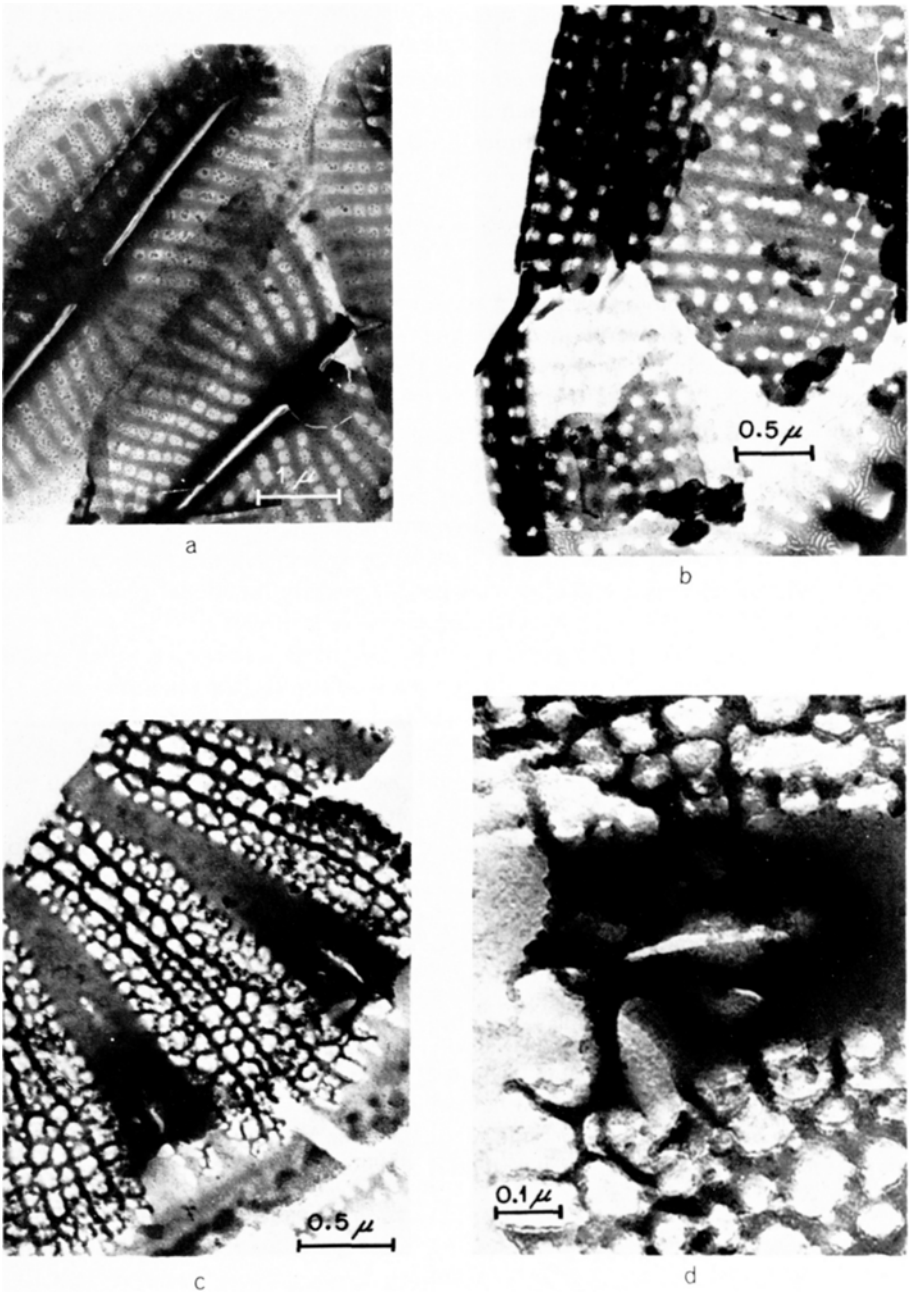


Fig. 47. Diatom cell walls after sonication and staining with phosphotunestic acid; (a) *Navicula pelliculosa;* (b) *Nitzschia brevirostris;* (c d) *Cyclotella cryptica* (Hecky *et al.*[548]). Around the circumference of the large pores, where there is no underlying silica, a bi-layer membrane can be recognized with grains showing an 80 A periodicity. For discussion of this periodicity see Ref.[48]

and species-specific. Relative to cell contents, cell walls are consistently depleted in glucose, while xylose is always higher in the cell walls. The ratio of amino acids to sugars is extremely variable 0.28 to 9.8. These variations may represent either real species differences in the constitution of the organic coating, or differences in the state of division of the different cultures[543] or the nutrient state of the culture[548].

(iii) Silicification

During silica deposition in diatoms a marked increase in protein concentration in cell walls is observed[543]; however, incorporation of carbohydrates increases only after Si-deposition. In addition, we have seen that there are consistent trends among specific amino acids in all analyzed species, while the sugars exhibit great variability. These two observations suggest that silicification is protein mediated[548].

The high content in hydroxyl amino acids (serine and threonine) may imply that hydroxyl-containing side chains are involved in silica deposition. Glycine, because of its small size may serve as a convenient spacer in the protein molecule. The notable reduction in the acidic residues between cytoplasmic protein and cell wall protein indicates that carboxyl groups are not the primary functional groups involved in Si-deposition. The following mineralization scheme is proposed[548]:

The inner surface of the silicalemma, *i.e.*, the limiting membrane within which silica deposition occurs[549] consists of a protein template enriched in serine and threonine. This protein will present a layer of hydroxyl groups which can undergo condensation reaction with silicic acid molecules with a consequent loss of water (Fig. 48). As a result, the initial layer of condensed silicic acid will be held fixed to the protein template of silicic acid (Fig. 49). Such a situation is kinetically more favorable than simply allowing the silicic acid molecules to come together by random collision.

Silica of diatoms closely approximates a silica gel, $SiO_2 \cdot nH_2O$. The degree of hydration varies among different diatoms species and determines its dissolution rates[550]. The amount of serine and threonine thus appears to be a controlling device in the degree of cross-linkage between silica and template, and the dehydration of the deposited silica. For siliceous sponges a similar silicification mechanism is proposed.

The role of sugars in the silicification of the cell wall is probably less direct. Amorphous silica is unstable in most natural waters, and is protected from dissolution in living organisms by the organic coating and, perhaps, metal ions[551]. Sugars are preferentially added to the cell wall after Si-deposition[543]. Assuming that these

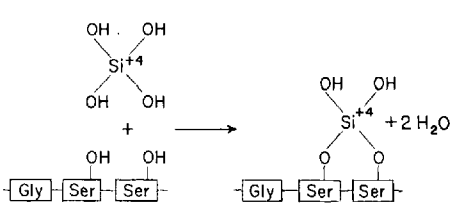


Fig. 48. Proposed condensation reaction between silicic acid and serine on the protein template of the silicalemma (Hecky *et al.*[548])

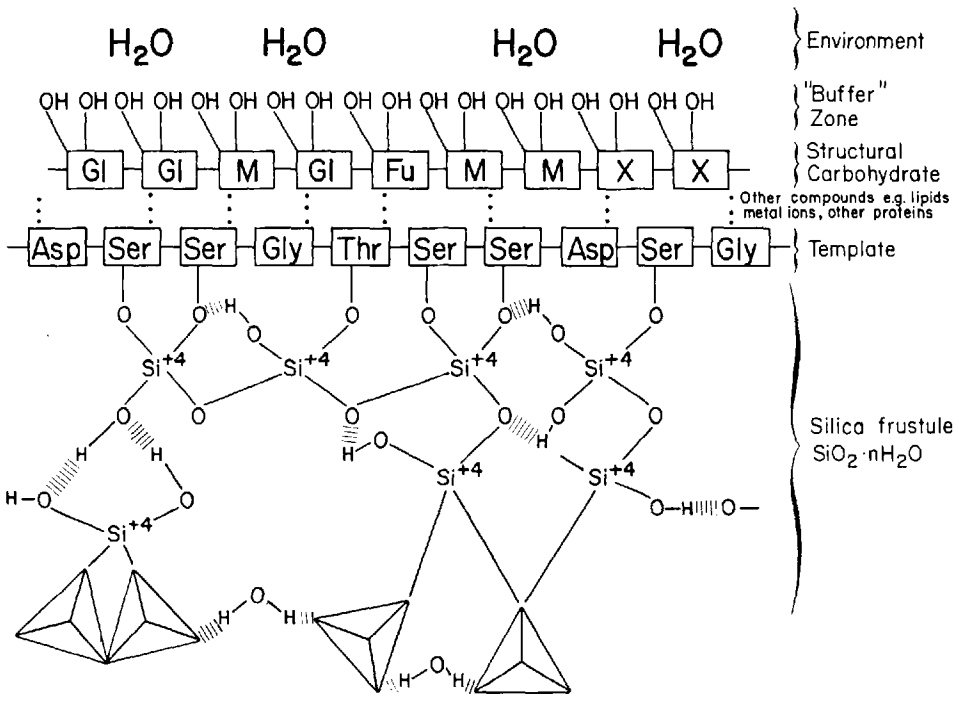


Fig. 49. Hypothetical arrangement of organic layers in the diatom cell wall; *Gl* glucose, *M* mannose, *Fu* fucose, *X* xylose. *Ser* serine, *Asp* aspartic acid, *Gly* glycine, *Thr* threonine: hatched lines represent hydrogen bonds (Hecky *et al.*[548])

sugars are largely present in the form of stable structural polysaccharides such as xylan, mannan, sulfated polysaccharides etc., they are probably more resistant to bacterial and chemical degradation than the protein coat which surrounds and interlocks with the silica after deposition. Also the hydrophilic nature of carbohydrates probably adds to the stability of the organic coating by providing a low energy "buffer zone" between the aquatic environment and the organisms (Fig. 49).

As is the case in carbonates and phosphates, silica deposition can be traced back to the Golgi apparatus where constituents of the cell walls are synthesized and ions are packaged for transport across the silicalemma. We have seen that protein-polysaccharides serve a carrier for Ca^{2+} ions, but in view of experimental data which show that Si-deposition procedes polysaccharide formation in cell membranes[543], it is questionable whether mucosubstances function as Si-carrier too. Instead proteins, or perhaps, lipoproteins appear to be more suitable Si-carriers and substrates in Si-fixation. Membranes of Golgi cisternae, and here in particular the surfaces composed of structural proteins, could provide sites for the coordination of Si^{4+} which is ultimately extracted from the endoplasmic reticulum. During transformation of vesicles into silicalemma, the sugars and uronic acids present on the inner walls of the cisternae become expelled to the outside and as a consequence protein and silica layers will turn upside down. Lipids and phospholipids will remain between the carbohydrate and protein layers and stabilize the membrane system through metal ion coordination[48].

(iv) Evolution of Biosilica

Present day ocean contains a few ppm dissolved SiO_2; diatoms are often cited as being the primary agent of silica removal from the worlds ocean and keeping this low $SiO_{2\,(aq)}$ level[552]. Others favor inorganic extraction[553], since it has been shown that common silicates take up dissolved silica from silica-enriched sea water, whereas silicates release SiO_2 into a silica-deficient sea water; in other words, silicates act as a buffering medium[554]. The data suggest that world ocean was low in silica for most of its history unless we assume that the environmental conditions in terms of pH or temperature have drastically changed through geological time.

The first siliceous fossils – radiolaria – are known from late Precambrian (?) or early Cambrian. However, most of the silica-extracting organisms have escaped fossilization because of the metastability of amorphous silica which reorganizes into cristobalite and/or quartz[555] during diagenesis. Chert beds are often the only remains of biogenic silica extraction from the hydrosphere. Precambrian sediments, especially since Gunflint time (ca. 1.9×10^9 years), are rich in bedded chert formations which frequently occur in association with iron ores (banded iron formations). The source for silica in these sediments could have been a biogenic one; alternately, the $SiO_{2\,(aq)}$ level in the Precambrian sea may have been much higher than to day allowing precipitation of amorphous silica[379].

Some recent findings have shed some light on the origin of silica in the Precambrian iron formations[556]. Alkaline effluents of hot springs promote the formation of algal and bacterial stromatolites which are almost entirely composed of amorphous silica. These stromatolites contain photosynthetic flexibacteria-conophyton-a group previously thought to have become extinct near the Precambrian-Cambrian boundary. The silica is precipitated nonbiogenically, due to cooling and evaporation of water. It encrusts the bacterial and algal filaments; microbial mats occur at temperatures up to about 70 °C. The principal components of Gunflint microbiota closely resemble the modern hot spring microbiota and siliceous stromatolites are known from the Gunflint too. It is quite likely that some Precambrian stromatolites were built by bacteria. Presence of stromatolites should not always be equated with cyanophytes or eukaryotic algae[556].

In view of these results it is propsed that carbonate and silica deposition in the Precambrian is an inorganic process, whereby in the case of stromatolite formation algal or bacterial mats generate a micro-enivornment for the organized growth of minerals. Siliceous plankton have been an important component of the oceanic silica cycle since the evolution of radiolaria during late Precambrian or early Paleozoic time[556]. In contrast, diatoms first appeared in the fossil record in the Jurassic. It has been suggested that the marked evolutionary trends in Cenozoic polycystine radiolaria are attributable to selection pressure resulting from the entrance of diatoms into the silica cycle.

In conclusion, cellular extraction of carbonate, phosphate and silica appeared almost simultaneously at about the Precambrian-Cambrian boundary. Which of these three modes of biomineralization is the most ancestoral one is not clear largely due to the metastability of some biominerals, the coexistence of different minerals within some primitive organism[557, 558], and uncertainties of stratigraphic correlation.

There is also the chance that calcareous, phosphatic, and siliceous fossil remains are products of metasomatosis.

X. References

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Water in Biological Systems

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Contents

"It is water that, on taking different forms constitutes this earth, this atmosphere, this sky, these mountains, gods and men, beasts and birds, grass and trees and animals, down to worms, flies and ants. All these are but different forms of water. Meditate on water!"

Thales von Milet

Is Life Mainly Inorganic Chemistry?

The well-known German poet Goethe once claimed: "If the eye were not sun-like, how could it perceive the sun? " Would he have said the same knowing as we do that eyes consist by 98% of water? Water is a *"conditio sine qua non"* for all biological systems. At medium humidity grain seeds can lie thousands of years without any sign of life, but under suitable humid conditions immediately germinate. At low water content irreversible denaturation of biosystems occur; for example DNA requires 30% of water in its native conformation of the crystalline state but partial dehydration leads to denaturation[1]. The important role of water in biological systems extends from the stabilisation[2–4] of the tertiary or quaternary structure by water to large enthalpy and free energy changes associated with chemical reactions involving the formation of water. Some marine vertebrates have a water content of 96–97%; the human embryo has during its first month a similar water content, *i.e.* 93% water, in agreement with the rule that the stages of the embryo growth are related to the evolutionary stages. The adult human consists of 65–70% water, nervous tissues 65–70%, liver 73%, muscle 77%, connective tissue 60% and adipose tissue 30%, plasma, saliva, gastric juices 90–99,5%[1]. 45–50% of the human organism is made up of intracellular water and 5% of plasma water[1]. An adult needs an intake of 2.5 liters per day of water in liquid form or with solid food (food consists of about 57% of water)[1]. (A further consideration is the transport protein of water in plasma and in plants.) – In organs we have to differentiate between the extracellular water (for example in canine ventricular myocardium, about 18 g water/100 g tissue[6] or about 50 g water/100 g tissue in canine cardiac Purkinje tissue[6]) and the intracellular water. Both differ in Na^+ and K^+ concentrations.

The structure of D_2O differs only in slight detail from H_2O, yet D_2O has a great effect on most organisms[7]. Mainly it is a poison or a retarder. In some cases organisms can be trained to grow with D_2O (algea, bacteria, yeasts fungi)[8]. This demonstrates that water functions in more ways than simply as a filling material. Its fine structure plays an important role in all biochemistry. There is no doubt that water is the most important material for all life processes. Without water there would be no life as we know it, but even so without the anomalous properties of water life could not exist on our planet: As a molecule water would be expected to melt at ca. $-90\,^{\circ}C$ and boil at about $-80\,^{\circ}C$[9]. This can be concluded from the discussion of intermolecular interactions, including such consequences as its boiling temperature

T_B or melting temperature T_M which are proportional to the molecular weights for non-polar molecules[9].

During the last 10 years biologists and physicians have recognized more and more the important role of water for biological and medical processes, but there are very few places where one can specify the exact role water is playing. In the last 15 years our knowledge of the water structure has grown considerably (see[10–12]), but still, our knowledge is incomplete. However, we have little knowledge of the structure of simple aqueous solutions and still less on more complicated aqueous systems[10–12]. Therefore, the applied water research of biological systems is only in its infancy, being based largely on assumptions[13, 14]. Some authors have recently assumed, that we have indications about water in cells existing in different states: bonded and unbonded ones[15–18]. The sparsity or our knowledge of the real influences of water on biologic processes is summarized in the Russian periodic series "Water in Biological Systems"[19] containing mainly articles on water structure problems and less on interactions water-biochemicals.

The main part of this article will be written by scientists in the near future. The goal of the paper is only to give a review on the present status of water research and on the first attempts to apply this to biological problems. Our main interest will be on the fundamental processes and not the complicated details such as membrane mechanism[20].

I. Structure of Water

a) Structure of Non-Polar Liquids

The structure of liquid water is one of the most complicated scientists have to unravel. But what do we know about the structure of simple liquids such as non-polar ones? Most university teaching courses neglect the structure of liquids although the greater part of their students of chemistry, biology, medicine or polymer science will later on work on questions of the liquid or amorphous state. The reason is that we should know the details of the partition function of molecular distances in a liquid and of the complicated mechanism of intermolecular forces in many body systems. The mathematics of this field of work is complicated[21–23] and the possibilities of proving the details of the theoretical assumptions are never satisfactory. Therefore, the effectiveness of this field of science on practical questions is not very large. In the research of liquids the second step has preceded the first one. It tried to find the exact theory before arriving at an approximate one. In the theory of gases the first step had been to get the ideal gas law with assumptions of molecules without any dimension and without any intermolecular forces. Even these assumptions have to ignore the partition of velocities of molecules or the properties of viscosity, of heat transfer, of diffusion or liquefaction. Nevertheless, the ideal gas model is needed very often as a good approximation.

It is also possible to give an effective model of unpolar liquids. The density of the liquids at low temperature T decreases linear with T at saturation condi-

tions[24–26] (there are only very few exceptions like H_2O). At higher T this straight line of linear density-decrease is the geometric locus of the sum of densities in liquid and vapour state at saturation conditions (see Cailletet-Mathias rule). This line can give the definition of an ideal liquid[24–26]. An ideal liquid is a liquid of which the density decreases linear with T by the heat movements of molecules. The ideal gas rules are more valid at high T, the ideal liquid rules are more valid at low T. At higher T (higher than the boilding T) there are real discrepancies with the ideal liquid behaviour. In a simple model one can assume these deviations are caused by hole defects in a real liquid. The concentration of holes can be given quantitatively by the

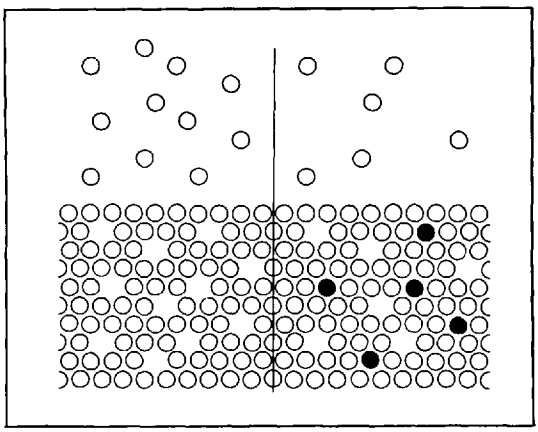

Fig. 1. Model of non-polar liquids. left: pure liquid: cristal-like arrangement of molecules with T-dependent vibration volume and holes. The hole-concentration is equal the vapour concentration. right: solution: solutes (black) are in the holes and reduce the vapour concentration proportional to the molar concentration of the solute.

concentration of vapour molecules. This very simple model gives a lot of liquid properties with errors of a few percent. It has a similar efficiency as the ideal gas model for the gas state[24–26].

The model presupposes a cubic densed package of molecules in a liquid. (Fig. 1 left). It includes a thermal vibration volume and T-dependent hole defects. The intermolecular interaction-energy of every molecule can be calculated by the sum of pair potentials of all immediate neighbours. This implies a cooperative mechanism which is typical for condensed phases. Co-operancy means: "The energy to produce a defect depends on the number of defects". In neighbourhood of a hole the sum of pairpotentials is smaller and therefore, the probability to produce a second hole is higher next to the first hole than in areas without defects. Co-operancy induces a cluster effect of holes.

The Raoult law, the decrease of vapour pressure p_s of a solution proportional to the solute concentration is a consequence of the model too. Solute molecules of which the own vapour pressure can be neglected have to be in the holes of the model (Fig. 1 right). Therefore, p_s of the solvent decreases corresponding to Raoult's law. Now this effect is reduced by the increase of the sum of pair potentials because the coordina-

tion number is increased by the solute. In consequence of stronger interactions the outer pressure decreases by the increase of the inner pressure. This pressure decrease will induce a solvent flow through a membrane until the osmotic pressure is in a state of equilibrium.

b) The H-Bonds

The hole model of a typical organic molecule is useful for all liquids with the exception of systems in which the H-bond interaction plays an important role. In this case the interaction becomes orientation dependant. This effect is most significant for liquid water. The degree of H-bond interaction on the total intermolecular energy is – in the case of water at room T – about 2/3 of the total energy (about 8 kcal/mol of 11.6 kcal/mol). Therefore, water is the most pronounced liquid of the H-bonded type. A large section of organic chemistry consists of molecules with H-bonds especially in biochemistry, pharmaceutical chemistry and plant protection chemistry. The properties of these molecules at room T depend definitely on the H-bond effects. Water is the most distinct example for studying the H-bond effects and an essential part of this important and interesting section of organic chemistry. It is clear that in mixtures water-hydrophilic organic molecules play a very important role.

There are some theories of H-bonds which claim the angle dependence of H-bond interaction. The interaction is maximal if the proton axe and the axe of the lone pair electron has an angle $\beta = 0$[27–29]. Neutron scattering results of crystal structure show in many cases a preference for this angle: $\beta = 0$[30], the H-O-H angle of H_2O is 105° in vapour state and 109.1° in ice[31]. All four charge centres of the two protons and the two lone pair electrons form theoretically the tetraeder angle of 109° with the 0-atoms as centre[31]. Neutron scattering results of hydrate structures show that the Y . . . O . . . Y angle between two H-bond acceptors Y and the O-atom of water is mainly centred about ± 15° deviations from the ice angle 109°. Figure 2 gives the

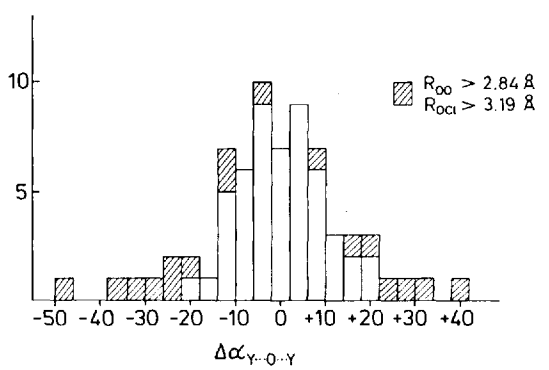

Fig. 2. Number of observed deviations of the H-bond angle zero in solid hydrates (Data are of the neutron scattering values taken from the review of Falk and Knop[32])

deviation $\Delta\alpha$ from the ice H–O–H angle for O and Cl aceptors (data-collection given by Falk and Knop[32]). The distribution of the O. . .O and O. . .Cl distances R of the same hydrates is plotted in Fig. 3. X-ray data of more than 400 hydrates show

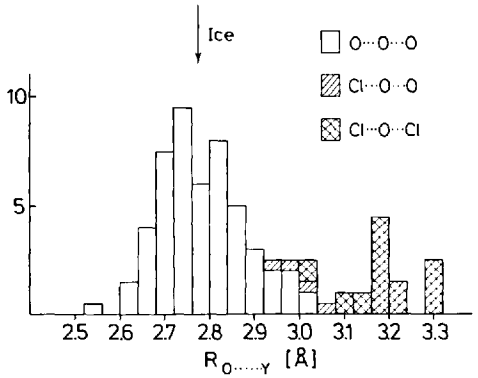

Fig. 3. Number of hydrates with O . . . O distances with a most probable O . . distance of about 2.8 Å (Data are neutron scattering values collected by Falk and Knop[32])

a maximum of the 0 . . . 0 distance[32] at 2.75–2.8 Å. The ice O. . .O distance is 2.76 Å[31]. A sensitive indicator for the H-bond interaction is the shift $\Delta\nu$ of the OH stretching IR frequency ν. $\Delta\nu$ is proportional to the H-bond energy ΔH[33–35]. (Badger-Baur rule[35]: $\Delta\nu = a \cdot \Delta H_H$). For alcohols in solution $\Delta\nu$ of the fundamental stretching vibration is about $\Delta\nu\,[cm^{-1}] \approx 0.1 \cdot \Delta H_H$ [kcal/mole][35]. For $R < 2.84$ Å $\Delta\nu$ is proportional too to R_{00}[36, 37] ($\Delta\nu = 425$ per $\Delta R_{00} = 0.1$ Å. For $R_{00}^{00} > 2.86$ Å there is an other linear relationship $\Delta\nu \approx 18\ cm^{-1}$ per $\Delta R = 0.1$ Å)[36]. There is no real H-bond from the IR spectroscopic view for O. . .O distances larger than 2.84 Å. Therefore, in Fig. 2 the data of hydrates in this O. . .O distance region are hatched. Hydrates with $\Delta\alpha > 20°$ belong to hydrates with more than 2 H_2O. They have a minimum of one H_2O with smaller H-bond angles and a second acceptor near Y as the cause of the angle disturbance[32]. Exceptions are $Li_2SO_4 \cdot H_2O$ and $Na_2Al_2Si_3O_{10} \cdot 2\ H_2O$[32]. The molecular H–O–H angle in the hydrates can bend in a region 102.5–115.5° with a mean value of 108°[32]. The base pairs of DNA form H-bonds with angle zero, the H-bonds of the amids groups of the α-Helix of proteins form $\beta = 0$ etc[29]. This orientation effect of the angle dependence of H-bond interactions is one of the most fundamental reasons for the specific effects in biochemical structures[27–29].

Figure 4 gives experimental evidence for this angle dependence of the H-bond energy. The part from $0° < \beta < 110°$ is the result of the IR matrix spectroscopy on H_2O and CH_3OH[12, 35, 38, 39]. The flatness of the curve near 110 °C is caused by the special arrangement of a cyclic "dimer-configuration" of Fig. 5b. Some quantum-mechanical calculations of dimers do not consider this configuration. One has to be careful with a generalisation of such calculations.

In the IR methods the frequency shift $\Delta\nu$ between the IR band of non H-bonded OH vibration and the different H-bonded, vibration is determined. By the Badger-Baur rule which gives a proportionality between $\Delta\nu$ and the H-bond interaction energy the points of Fig. 4 are[12, 35] obtained. The curve goes down symmetrically to zero at 220°. This curve is taken in the energetically most favoured plain of two molecules. In other plains there are different curves with repulsion orientation as well.

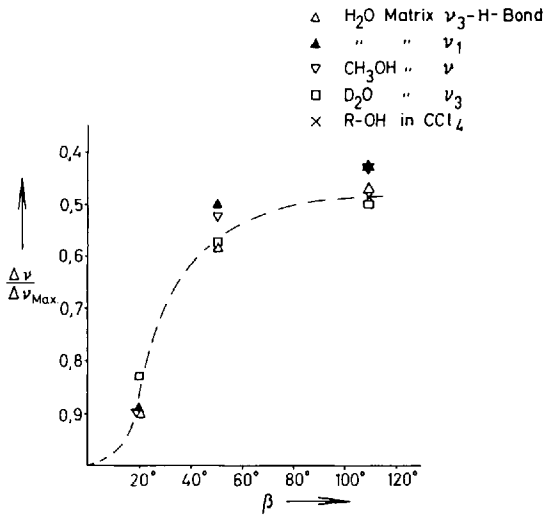

Fig. 4. The frequency shift $\Delta\nu$ of the OH stretching mode over the maximum $\Delta\nu$ in linear H-bonds as a function of the H-bond angle β. This figure also gives the angle dependence of the H-bond energy ΔH ($\Delta H \approx \Delta\nu$)

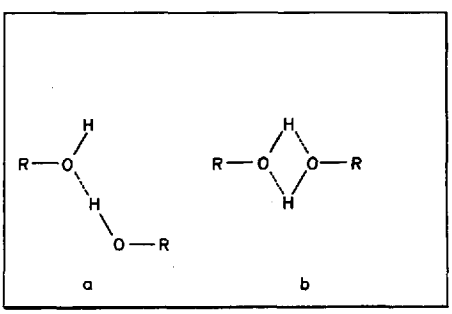

Fig. 5. a) linear H-bond; b) cyclic H-bond dimer

With larger angles we have to expect repulsions if proton-proton or lone pair electron-lone pair electrons are coming nearer. The flat minimum of Fig. 4 till $\beta <$ 20° is in agreement with the angle distribution curve of hydrates in Fig. 2.

The Fig. 4 shows the preference of H-bond angles zero. This effect determines many biochemical structures and the C_{2v}-symmetry of the ice lattices in which every water atom is surrounded by four neighbours (see Fig. 5 in Ref.[31]). The angle dependence of H-bonds with preference of $\beta = 0$ is the cause of the small density of ice with holes between the six membered rings of H-bonded water molecules. Similar curves are valid for H-bonds of other organic molecules[12, 28]. The C_{2v}-symmetry dominates the structure of neighbours in liquid water till 200 °C. This is known from Raman data[40] and the X-ray scattering of liquid water[41].

In addition to the hole defects in a liquid with H-bonds the angle dependence of the H-bond interaction induces an orientation defect mechanism. Figure 4 gives indications on co-operative mechanism of orientation defects as well.

If there is a hole defect in a lattice or lattice-like arrangement of water molecules, it will form a 5 membered ring instead the 6 membered ring of the tridymite-like structure with $\beta = 0$. A 5-membered ring will have β about 10°. That means the interaction to produce an orientation defect depends on the number of defects. But the orientation effects are only one parameter of aqueous mixtures. A second parameter is the dependence of the H-bond interaction energy on the base strength of the lone pair electrons of hydrophilic organic molecules. For example the frequency shift of the OH stretching vibration of water as measure of the H-bond interaction increases in the series[31, 42]: nitromethane, nitrobenzene, trioxan, dimethylphthalate, 3,4-dimethoxybenzaldehyde, acetonitrile, butyrolacetone, ethylacetate, methylale, acetonedioxan, t-butylperoxide, ethylether, N,N-dimethylformamide, pyridazine, pyridine, 4-picoline-N-oxide, etc. (Fig. 34 in[35]).

c) The H-Bonds in Water

A review of the past knowledge of the water structure is given in this journal[31]. Meanwhile the point of view reflected in this report has become more pronounced. During the last years various attempts were made to calculate a model of liquid water by simulations of water interactions by computer models[43, 44]. The result has been that there are in water preferences of certain interaction energies which are related to the preference of certain angles. But the computer results of the so called molecular dynamics method depend strongly on the assumed angle dependent interaction potential[44, 45]. A point charge model taken by Rahman and Stillinger[46] gives one distinct maximum for the distribution function of H-bond energies at an energy of 5 kcal/mol[46] or 4 kcal/mol[47] and a second shoulder at 2.44 kcal/mol[46]. Ben-Naim tried a plain model with a charge distribution like a Gaussian function. He got 4 different maxima at 4 different interaction energies[48]: 2.7 ΔH_H; 2 ΔH_H; ΔH_H and 0.2 ΔH_H (ΔH_H: H-bond energy). At room T the main part of molecules is in a complete H-bonded configuration. By the results of Fig. 4 we could expect certain preferred different species, cyclic 6 membered rings with $\beta = 0$; cyclic 5 membered rings with $\beta = 10°$, cyclic 4 membered rings and so on until we arrive at cyclic dimer rings with $\beta = 110°$. The IR matrix technique does not recognize a continuum of β-values but 5 more or less sharp H-bond bands[35, 39]. This means that there are about 5 different preferred H-bonded arrangements in water. The flatness of the curve of Fig. 4 at $\beta = 0$ and $\beta = 110°$ gives a higher probability for these two β regions. The interaction energy for $\beta = 110°$, is about half of the energy for $\beta = 0°$. This may be related to Rahman and Stillinger's results of two preferred energy regions, about 5 kcal/mole[46] respectively, 4 kcal/mole[47] and 2.5 kcal/mole[46]. In addition the IR method can alter distinctly the bands of non H-bonded OH groups in liquid water[35, 49, 50]. The IR overtone spectra can determine quantitatively the content of non H-bonded groups[49, 50]. The IR fundamental bands of water[51] don't give good possibilities to determine the H-bond content by overlapping effects. For this reason some confusing results are given in literature about mid. IR methods. In addition the IR method indicates a distribution of different H-bonds as a result of the distribution of different angles and different OH . . . O distances. The X-ray scattering

methods[41] show that the distance distribution is not large, therefore, I am inclined to assume that the angle distribution plays the dominant role. The spectra show in addition that angle distributions have some crucial points (compare the matrix results). A computer analysis of the bands[50] needs only three different species with a certain distribution: the non H-bonded OH groups, the linear H-bonds with ice like $\beta = 0$ and H-bonds with intermediate angle about 110°. The spectra of liquid water give as function of T more or less sharp isosbestic points for $T < 150$ °C[49, 50]. This is an indication that two species dominate the T-dependent equilibrium: the non H-bonded and the linear H-bonded groups. Our computer analysis of the better resolved HOD spectra show that the concentration of the third species with $\beta \approx 110$ °C gives a broad maximum about 60 °C which is not a strong T-dependent[49, 50].

The H-bond interaction energy of the linear H-bonded OH groups in water is about 4 kcal/mol[49, 52] [a]). Some lower values given in the literature are erroneous from our point of view[49].

Because the computer models of the water structure are sensitive to the assumed angle dependence of the interaction potentials this method cannot give today an exact theory of water structure[44, 53, 54]. The result of the Rahman-Stillinger[46] calculation does not give good values for the specific heat and gives doubtful results on the pressure dependence of the water structure[55]. There are two goals of water research today: 1. To search for better methods to get the exact partition function of orientation and hole defects in water and aqueous solutions; 2. Intermediately to search for useful approximation models for the applied research. The goal of this paper is to show that some spectroscopic results give models which are surprisingly efficient to comprehend different properties of aqueous systems with common principles. One of the best methods to get useful approximations is the IR overtone spectroscopy. At first we can get approximate values of the content of non H-bonded OH groups of water and alcohol (Fig. 6). Figure 6 shows that the co-operativity of

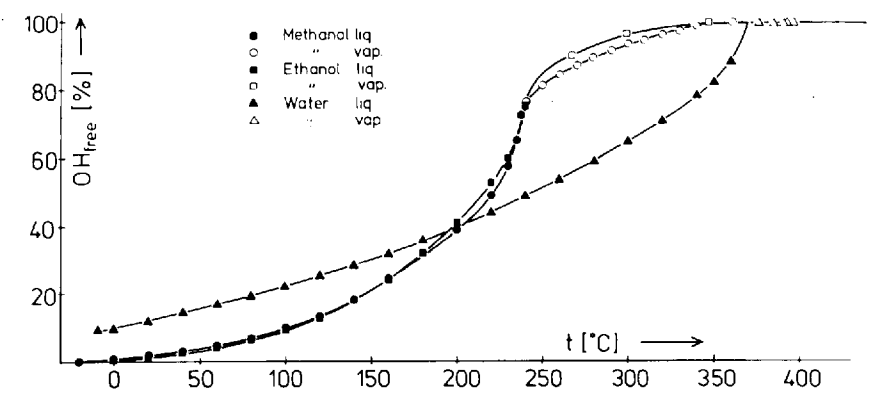

Fig. 6. The percent of non H-bonded OH groups in liquid water and alcohols under saturation conditions. (Determined by the IR overtone method[49])

[a]) (s. Kochnev in[19] Vol. III, p. 9).

the network of water H-bonds is much higher than in alcohols which have more chain-like H-bonded structures. Therefore, at the melting point T_m the break of H-bonds in waters is 10 percent higher than with 1 percent in alcohols[49, 56]. To get an efficient approximation of the water structure it is necessary to study the water structure till the critical temperature T. Most of the approximation theories have not done this and adjust assumed constants for $0\,^{\circ}C < T < 100\,^{\circ}C$. In consequence of this proof most of the water theories which view only a small T-region until 100 °C, made erroneous approximations and got too large contents of non H-bonded OH groups (complete orientation defects O_F). These theories are in contradiction with the experimental curve of Fig. 6. We have to stress the assumptions[49] of our approximation method to get Fig. 6, it is in this direction that the real orientation defects may be less than our values. Therefore, the discrepancies between the theories and the real structure may be even larger than Fig. 6 shows. For one of the best known approximation theories of water, the so called Némethy-Scheraga theory[57], the authors have recently given themselves[58] a collection of the "Non validity" of their older assumptions.

The overtone IR method gives good approximation values of the non H-bonded groups. These are one end of the distribution function of angle orientation in the H-bonds. Getting informations on the different angles in closed H-bonds is much more complicated by the splitting of the water valence vibrations in a symmetric and an asymmetric one, by Fermi resonance and the overlapping of the H-bond bands corresponding to different angle orientations. Some work on these problems has to be done in future. As a first step we can try to give a very rough first approximation to substitute the partition function with a two step function: non H-bonded OH groups and bonded ones. The overtone and the Raman spectra require a minimum to take a three step function: non-bonded, linear bonded OH and H-bonded groups with medium bond angles[50]. From the result the third step of the intermediate angle shows up to 40 °C only a small T-dependence is the first approximation of a two step function, this can be reasonably assumed for biologic purposes. Surprisingly this two step function is efficient[49, 59] to describe some water properties in a large T-region till 200 °C.

An analysis of the IR fundamental water bands is more complicated. But experienced authors agree with the overtone results very well. The papers on band analysis of the OH stretching fundamental band given by Mikhailov and Zolotarev[19] (Vol. 3, p. 15.) is consistent with our analysis of the HOD 1. overtone stretching vibration[50].

Fundamental[19] ν_1 [cm^{-1}]	ν_3 [cm^{-1}]	1. Overtone[50] $2\nu_1$ [cm^{-1}]	Coordination[50]
3620	(3720)	7150	Non H-bonded
3420	3490	6850	Centrum of unfavourable H-bonds (angle, distance)
(3280) 3280	(3320)	6400	Linear H-bonds
$3080 = 2\nu_2$			See Kochnev and Sidorova too (in[19] Vol. III, p. 81)

d) Water Properties

1. Density

The T-function of the density including the density maximum at 4 °C can be described by the Eq. (1)[59]:

$$\frac{1}{\rho}(\mathrm{cm^3 g^{-1}}) = (1 - O_F)\,[1.035\,(1 + 1.741 \cdot 10^{-4} \cdot t(^\circ\mathrm{C}))] + O_F\,[0.64006\,(1 + 5.7249 \cdot 10^{-3} \cdot t(^\circ\mathrm{C}))] \quad (1)$$

The first part of the right side of Eq. (1) gives the portion of the H-bonded OH groups with the concentration $(1–O_F)$; the second part gives the portion of the non H-bonded OH groups with the concentration O_F. The partial molar volume of H-bonded groups and the coefficient of thermal expansion is taken as ice like datas. Both properties of the orientation defects are adjusted. Spectroscopy cannot give informations on these constants. Therefore, the proof of the orientation defects assumption by the density is not very accurate.

The density maximum at 4 °C is induced by the sum of two effects. The normal lowering of density with increasing T by the increase of the volume of thermal vibrations and the rise of ρ with T by an increase of the content of O_F. The structure near the orientation defects is more like non-polar liquids with their densed package and higher coordination number.

The T of the density maximum is decreased linearly by the concentration of added salts (Despretz effect[60, 61]). The constant of molar decrease depends on the type of ions[31]. There is a distinct but small difference in this constant between Na^+ and K^+-ions. Large anions like I^- or NO_3^- give bigger effects than small anions like Cl^- or Br^-. The Despretz effect can be understood by the assumption that the ions destroy the loosely packed ice like H-bonded structure near 0 °C.

2. Specific Heat

The specific heat C of water can be calculated by Eq. (2)[59]:

$$C = C_{v,v} + \frac{dO_F}{dT}\,\Delta U_H + 2(1 - O_F)\,R \quad (2)$$

$C_{v,v}$ is the specific heat of water vapour in the ideal state, it is determined by the intramolecular degrees of the water molecule freedom; the second term in Eq. (2) gives the change of the content of orientation defects by change of T and the third term gives the effect of intermolecular degrees of freedom in all liquids[24]. ΔU_H gives the H-bond energy, it is taken as 4 kcal/mole[49, 52]. Equation (2) includes in contradiction to Eq. (1) no adjusted constants. dO_F/dT is got by the experimental results of Fig. 6. Eq. (2) gives a sufficient agreement with the experimental T-dependence of the specific heat and its absolute value[59]. It is able to show the reason for the minimum of the specific heat at 36 °C (Fig. 7)[62, 63]. dO_F/dT, the change of the orientation defects per degree is nearly constant in the region $0\,^\circ\mathrm{C} < T < 35\,^\circ\mathrm{C}$

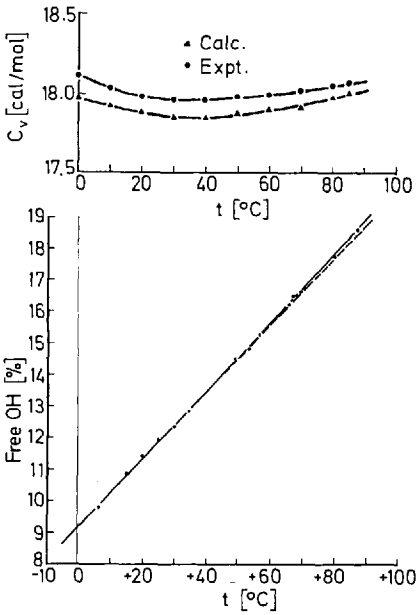

Fig. 7. lower: A section of Fig. 6 with enlarged scale. upper: The experimental values of the specific heat of water and these calculated by the IR overtone method

(Fig. 7 above), but the third term in Eq. (2) decreases with T. Above $T = 35\,^{\circ}C$ dO_F/dt is increasing. This may induce the minimum of C_v at 36 °C.

The correlation between the Fig. 6 and the heat content H of water is clearly seen by the comparison of the fraction of liquid structure with the enthalpy change ΔH by heating water from 0 °C till 100 °C:

$$\Delta H = C_p(\text{liq}) - C_p\ (\text{vapour}) \cdot T =$$

$$(18 - 9)\,(\text{cal/mol}^{\circ}) \cdot 100^{\circ} = 1\ \text{kcal/mol}.$$

Figure 6 shows:

$$O_F(100\,^{\circ}C) - O_F(0\,^{\circ}C) = 0.12.\ \text{It is}\ 0.12 \cdot 2 \cdot \Delta H_H = 0.96\ \text{kcal/mol}.$$

Table 1. Specific heats of liquids

	C_p (cal/Mol · °)	C_p (cal/g)	t °C
H_2O	17,99	0,999	20
CH_3OH	18,4	0,57	20
C_2H_5OH	25,7	0,56	20
CH_3-CO-CH_3	29,7	0,51	20
C_2H_5-O-C_2H_5	41,1	0,554	20
$CHCl_3$	26,9	0,22	20
CH_4(g)	8,48	0,53	20
C_2H_6	17,1	0,57	−100
C_3H_8	23,1	0,52	− 53

A comparison of C_p of different materials (Table 1) shows that the specific heat of water per gram is abnormally high. The high value of the second term in Eq. (2) by the H-bonds induces high C_p values of water, although the intermolecular degrees of freedom are low. The smallness of the water molecule influences in addition the big values of C_p per gram. The large values of C_p of water have an effect on the thermostat function of organism. It is easier to construct a good thermostat with a large heat content. For animals it is efficient to have a large heat content with low weight. That means C_p per gram should be large.

3. Heat of Melting

The heat of melting ΔH_M of ice is 1.43 kcal/mol. The influence of the dispersion forces at ΔH_M can be assumed by ΔH_M of H_2S. Therefore, the change of O_F at melting can be estimated by[31]:

$$O_F = \frac{\Delta H_M(\text{ice}) - \Delta H_M(H_2S)}{2\,\Delta H_H} = \frac{1.43-0.57}{2 \cdot 4} = 10.7\%$$

The overtone IR results give[49]:

$$O_F\,(0\,^\circ\text{C}) \approx 10\%$$

4. Heat of Vaporisation

The heat of vaporisation L_v of water can be described in a good approximation with Eq. (3)[59]:

$$L_v + W_{real} = (1 - O_F)\,\Delta U_s + O_F W - 2\,(1 - O_F)\,RT \qquad (3)$$

W_{real} is the energy to transfer the real vapour state to the ideal gas state. W_{real} is dependent on the vapour pressure and therefore on T. It can be neglected for $T <$ 100 °C. The first term of the right side of Eq. (3) gives the energy to transfer 1 mole of H-bonded OH groups to the ideal vapour state (ΔU_s = sublimation energy of 11.64 kcal/mol as measure of the total interaction energy of the bonded species). The second term is the energy needed to transfer 1 mole of non-bonded groups to the ideal vapour state. W = 3.6 kcal/mol is the van der Waals interaction energy of water molecules (non H-bonded interactions). The third term gives the heat content of intermolecular degrees of freedom.

L_v is the heat of vaporisation at constant volume. The heat of vaporisation at constant pressure $\Delta H_p = L_v + p\Delta v$ is given in Table 2 for different molecules. The table shows that ΔH_p per gram is abnormally high for water. The cause is the large value of the first term in Eq. (3) and the smallness of the water molecules. The high values of ΔH_p for water are important for the efficient cooling mechanism by vaporisation of perspiration of animals.

Table 2. Heat of vaporisation of liquids

	ΔH (kcal/Mol)	ΔH (cal/g)	t °C
H_2O	10,5	585	20
CH_3OH	9,4	287	20
C_2H_5OH	10,1	240	20
CH_3-CO-CH_3	7,4	131	20
C_2H_5-O-C_2H_5	6,4	86	34
$CHCl_3$	7,5	63	20
C_2H_6	2,2	75	0
C_3H_8	3,6	83	20

5. Surface Tension

A further abnormal property of water is the abnormal high surface tension σ. The abnormal high values of water depend on the large H-bond interaction energy of water but in big matter from the smallness of the water molecules too. σ gives the energy to enlarge the surface by 1 cm^2. The number of molecules per cm^2 surface is higher in water than in organic molecules. To discuss structure effects one has to calculate σ_M, the energy to enlarge the surface by one mole[24, 64] $\sigma_M = N_L^{1/2} V^{2/3} \sigma$. σ_M of water is anomalously high too, for example it is larger than σ_M of ethanol or methanol, but it is not larger than σ_M of large organic molecules like benzene or carbonic acids, soaps and so on.

σ_M is a free energy in the nomenclature of thermodynamics. The surface energy H_σ can be calculated by σ_M by the Gibbs-Helmholtz equation[24, 64]:

$$H_\sigma = \sigma_M - T \frac{\partial \sigma_M}{\partial T} \tag{4}$$

H_σ is T-independent[24] for non-polar liquids for $T/T_c < 0.9$. But it has a maximum for H-bonded liquids. The value of H_σ and its maximum can be calculated by the spectroscopic determined O_F-values too[59]. The large value of σ gives the possibility that water ascends high in capillaries of tall trees.

6. Association-Structure

The possibility to calculate the anomalous properties of water quantitatively with a simple two step function of the orientations partition function of water molecules gives the possibility to estimate the size of the network of H-bonded molecules in liquid water too. We have to take into account the cooperative mechanism of H-bonds and the preference of C_{2v} symmetry of intermolecular arrangements in

water[b]). If we can assume aggregates of water in ice-like arrangements and assume that the orientation defects are concentrated at surfaces of such aggregates, we can estimate the mean-size of these idealized aggregates by the spectroscopic values of O_F[12, 49, 50, 59]. The cluster size is at room T about 240 at 37 °C about 150 at 45° about 120 at 100 °C about 40 molecules.

The dimension of the network of H-bonded molecules in liquid water decreases exponentially with T and an energy of 5 kcal/mol[49].

The summary of our simple approximation model of water is: water consists at $T < 100$ °C of a network of H-bonded molecules. The co-operative mechanism of the angle dependence of H-bond energy has the consequence that the complete orientation defects of non H-bonded OH groups are not distributed statistically, they are concentrated by fissure plains of defects. The content of orientation defects at room T is about 12%. The number of molecules per idealized aggregate at room T is about 300 molecules (about 7 in one dimension).

Fisher ([19] Vol. III, p. 24) has calculated the thermal fluctuations in the polarisation vector of water. He obtained a correlation radius at 0 °C of 14 Å. This would correspond to a trydimite like aggregate of about 700 molecules and 10 percent orientation defects (compare this result with the IR overtone method). We have to stress that the spectroscopic method just gives information that the life time of such aggregates is larger than 10^{-14} sec. There are other methods like the dielectric constant[65] or NMR measurements which indicate a relaxation time in water of 10^{-12} sec. This should be the lifetime of orientation defects too. In this order of time-intervals the orientation defects are moving through the liquid. During this moving mechanism intermediate orientations of different angles of H-bonds may occur. We have to stress one important result of the overtone IR methods: The small content of non H-bonded molecules (10% at 0 °C and 22% at 100 °C) makes it improbable to assume a noticeable content of monomers (water molecules with two non H-bonded OH groups). If there were a larger amount of monomers for instance, like a lot of models of liquid water assume, the vapour pressure of water should be much higher and the boiling point should be lower, the surface tension should be smaller etc. Rahman and Stillinger[46] remarked: The molecular dynamics calculation conflict with those "two-state theories" of water which divide molecules dichotomously into "bonded" versus "unbonded". This conclusion was arrived at the calculation of the numbers of H-bonds per molecule[46]. But one has to pay attention to the fact that this conclusion is not valid for a model with bonded and unbonded OH *groups*. The statement of Rahman and Stillinger[46] is in agreement with the overtone IR method with the accentuation that we cannot assume unbonded *molecules* and bonded ones, but just bonded and unbonded OH *groups*.

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[b]) Sidorova and Mioseeva conclude on the similar H-bonds in liquid water at the melting point and ice from the view of the equal frequency of the 700 cm^{-1} F.I.R. libration band. In liquid water at higher T this frequency is sensitive to T, (s.[19] Vol. II, p. 69) by increasing intensity of a second libration band of the liquid state at 500 cm^{-1} (s. Moiseeva and Braginskaya in[19] Vol. III, p. 11). We have found[39] with the matrix technique a "polymer-band" at 675 cm^{-1} and an isolated band of small aggregates at 525 cm^{-1}. This result established the analysis of the two overlapping bands given by Moiseeva *et al.*

The spectroscopic results show that the IR frequency of one non H-bonded OH group in water changes only little whether the second OH group is H-bonded or not. The frequency shift is only 8% of the shift of the bonded group[49]. This indicates that the cooperativity effect on the second OH group by H-bonds – as is reported in some papers – is very small. The cooperativity effect of the angle dependence of H-bond energy is much higher. From this experience we would expect that the chemical reactivity of a non H-bonded OH group in water does not change much if the second OH group is H-bonded or not.

II. Structure of Aqueous Solutions

a) Electrolyte Solutions

There are very few methods for obtaining detailed knowledge on the structure of electrolyte solutions. The Debye-Hückel theory of very diluted solutions is simplified, it ignores the water structure and gives wrong data on the specified heats. At higher concentrations we know sensitive salt-out effects on the solubility of organic molecules in water by ions. Some ions like perchlorate or jodide can give salt-in effects. The overtone IR spectra give some indications for the structure of electrolyte solutions at medium concentrations too. The spectra of electrolyte solutions show the following results in head lines[12, 49, 66]:

1. The water spectra are relatively insensitive to added salts. We need concentrations larger than 0.5 mole/liter at room T to observe distinct changes of the water spectra. The sensitivity of the water spectra on ions increases with T.

2. In the first approximation the changes of the water spectra by added ions is similar to a T-change of pure water. Once Bernal and Fowler[67] have introduced quantitatively the nomenclature structure temperature T_{str} of electrolyte solutions. T_{str} is the temperature of pure water with a similar H-bond content as the solution. The IR method gives a possibility to determine T_{str} quantitatively.

3. The ion series gained by change of water spectra determined by T_{str} is similar to the lyotropic ion series or so called Hofmeister ion series according to these observations. The cause of the lyotropic ion series is identified as an effect on the water structure. The salt series in the order of changing the water spectra is:

$$MgSO_4 > Na_2CO_3 > (NH_4)_2SO_4 > Na_2SO_4 > MgCl_2 > BaCl_2 > NaCl > KCl > KBr > NaJ > KNO_3 > NaSCN > KSCN > KJ > NaClO_3 > NaClO_4 > Ba(ClO_4)_2.$$

This series concerns spectra at 20 °C and anion concentrations C_A = 1 mole/l. The series changes a little with T and C_A.

4. The main effect on the water spectra is determined by the type of the anions, the cation effect differs less than the anion effect.

5. There are some ions with an effect on the water spectra like a T-reduction, these ions have the largest salt-out effect on the solubility of organic molecules in

water. These ions are called structure-maker. The spectra of aqueous solutions with such ions show a smaller content of O_F; the possibilities of interactions with hydrophilic molecules is reduced.

6. There are some ions which effect the water spectra like a T-increase. Ions with the largest "structure-breaker" effect can have salt-in effects on organic molecules. This can be understood as follows: the water becomes more hydrophilic because the content of orientation defects of OH groups increases. Structure breakers are mainly large mono-valent anions.

The smallness of the ion effect on the water spectra is contradictry to the large effects by ions on the solubility of organic molecules in water. The apparent paradox can be easily understood by the simple water model. At room T the content of orientation defects is not very sensitive to the expansion of H-bond systems[12, 49]. At higher T the expansion of the system of H-bonded molecules decreases; one can calculate that the sensitivity of the content of orientation defects on a change of the

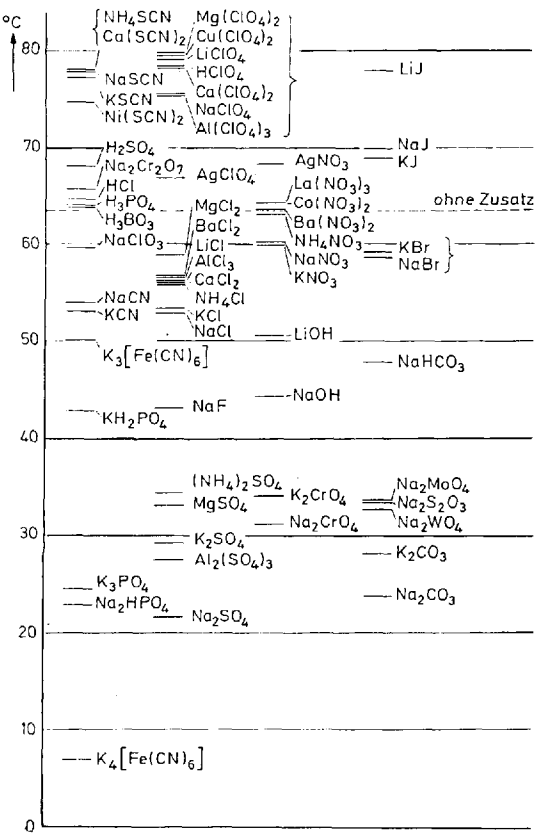

Fig. 8. The turbidity points T_K of aqueous solutions of C_8H_{17}-⟨⟩$(-O-CH_2-CH_2-)_9OH$ (PIOP-9). (10 g/l) with 0.5 m salt additions. T_K without ions: 64 °C. The figure shows the Hofmeister ion series similar to the influence of these ions on the water spectra and structure

dimension of H-bonded molecules is larger at higher T in agreement with our spectroscopic observations.

The Hofmeister ion series can be determined easily by the turbidity points T_T of aqueous solutions of $H_{17}C_8$-⟨⟩-$(O-CH_2-CH_2-)_9OH$(PIOP 9) (see. Fig. 8). T_T in pure water is 64 °C. The Fig. 8 shows the boundary between structure makers (lower T_T) and structure breakers (higher T_T), these are the nitrates. NaCl and KCl show a small but distinct difference. The boundary between structure makers and breakers depends on the organic solute[31]. In pure water this boundary is a function of the temperature. The influence of NaCl on the water structure is smaller than the greater part of other salts. Its property as small structure breaker at room T changes to a structure maker at higher T. Figure 8 shows distinctly that the salt effects on aqueous solutions – and on the structure of liquid water – depend on the type of ions. NaCl shows relatively small effects. The change of the turbidity points of PIOP 9 depends on the quotient of the charge e and the radius of the anions r_{An} like e^2/r_{An} (Fig. 9).

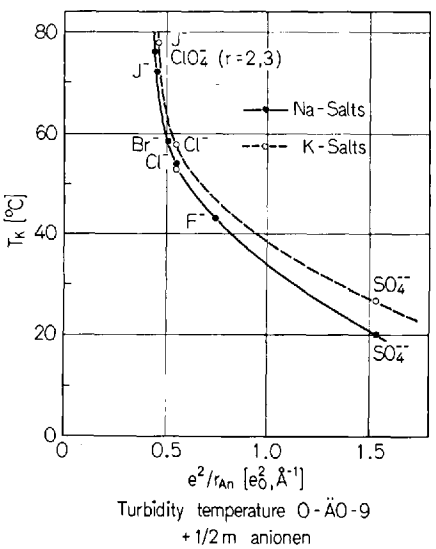

Fig. 9. The turbidity T of PIOP-9 as function of charge ^{2} over radius of the added anions[224]

The effects on T_{str} of pure water are similar. The dependence of the radius of the cations r_{Kat} is smaller (Fig. 10).

The ion effects can be understood by the assumption that the electric field of the ions strengthens the orientation of water dipoles by H-bonds. But the large anions disturb the long range orientation of the H-bond system. At higher T the structure-breaker effect of the large anions is weakened and the electric field effect dominates. The field strength effect is observed too by the influence of ions on the dielectric constant of water. Debye[68] and Sack[69] have calculated by this dielectric effect with a continuum theory, that around every ion with one charge all water dipols are fixed at a distance of 11 Å. In the ice lattice a radius of 11 Å includes about 400

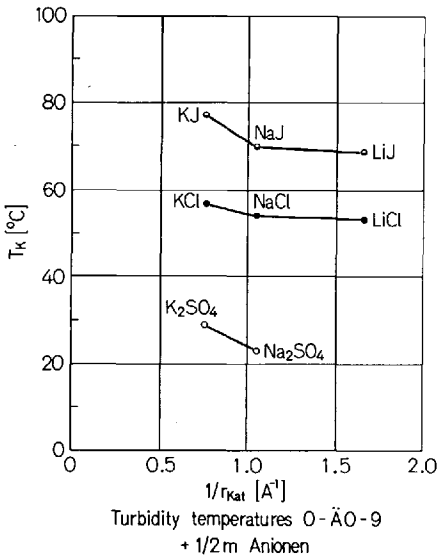

Fig. 10. T_K of PIOP 9 as a function of charge ^{2} over the radius of added cations[224)]

water molecules. Azzam[70)] has calculated with regard to the water H-bond structure that around a single charged ion the dielectric constant at a distance of 6 Å is reduced to 40. In a sphere of this radius there would be about 100 water molecules. A reduction of T_{str} of 10 °C by 1/2 mole $MgSO_4$ means at room T an increase of the mean number of water molecules per aggregate of 100 molecules. In a model we can assume an ordered sphere of secondary hydrates of 100 water molecules around the ions and a continuation of this ordered area by the normal water structure about 500 molecules.

If we calculated with the idealized co-operative model by the content of spectroscopic determined O_F values the number N_{el} of H-bonded water molecules we would get – with different 1 molar salt solutions – the result of Fig. 11. The values N_{el} with salt additions depend strongly on the salt concentrations because of the disturbance of the big H-bonded system[31)]. At small concentrations the $N_{el}-N_0$ numbers (N_0 association number in pure water) of structure-makers are in size of the order of Debye-Sack's or Azzam's calculations. They are of the same size of order as the secondary hydration numbers calculated by solubility measurements of organic substances in water (Chapter b) or as the hydration numbers of hydrophilic organic molecules (Chapter IId–e) or biopolymers (Chapter III).

Negative values of $N_{el}-N_0$, the electrolyte effect on the association numbers of water, are called the structure-breaker effect. One can speak of negative hydration[31)]. The estimation of the hydration numbers by spectroscopic or solubility methods gives only an approximation of the sum effect. The spectra of the H-bond bands show in second approximation distinct differences between the ion effects on the H-bonds[71)]. – The partial molar volume V_1 of water in electrolyte solutions is negative in all solutions but the series of V_1-values corresponds to the Hofmeister ion series too. The negative V_1 volume indicates an electrostriction effect around the ions.

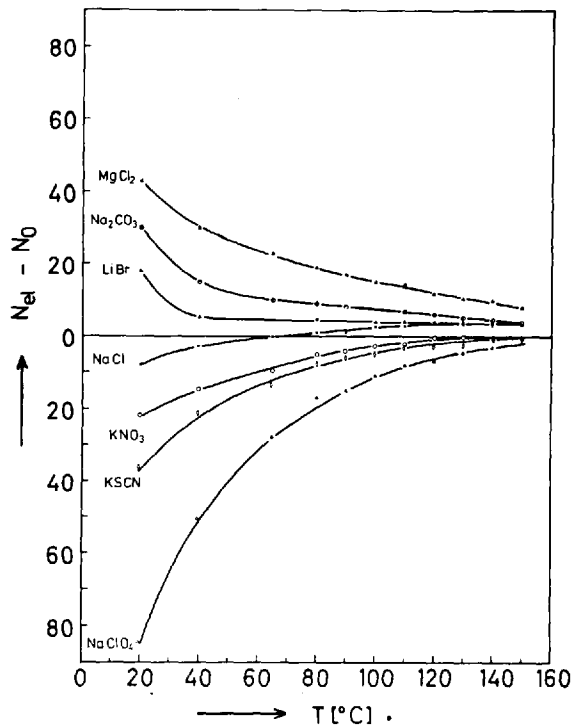

Fig. 11. The spectroscopically determined change of the extension of the H-bonded water molecules from 0.5 m added ions as a function of the T

Aqueous solutions of acids or bases have a large effect on the half width of the IR bands[31, 72–74, 76]. This may be influenced by the shortness of the life time of H-bonds[31]. At high acid or base concentration the IR band changes to a continuum. Zundel[77] has discussed a tunnel effect as cause of this continuum. The quick exchange-processes of excess protons of H_3O^+ or "defect-protons" of OH^- with surrounding water molecules induce the dissociation processes of acids and bases[81].

The structure of aqueous solutions depends on the following parameters: 1. content of H-bonds of water molecules, 2. interactions water-solute, 3. orientations of H-bonds, 4. H-bond distances, 5. life-time of H-bonds, 6. movement of protons in acids or "defect-protons" in bases. There are some computer experiments with water and a model of ions[78]. They show a separation of the ions in 1 psec but that the distance of ions remains about 5 Å[78]. This gives a simple interpretation of the optical constancy of absorbing ions independantly from the concentration[79].

b) Non-electrolyte Solutions with Hydrophilic Solutes

The solubility properties of water depend on many parameters. Today it is not possible to give a complete theory of aqueous solutions and mixtures. We are contending the attempt to make the head lines classified with a hypothetical model. Starting

from observations of the concentration dependence of the solubility co-efficients of gases in water Schröder has given[80] a simple theory of solubility in aqueous solutions. He assumes two different solubility mechanisms of two different energetic states of the cluster surfaces (corners and plains). At higher concentrations of the solute there may be a third mechanism accompanied by a disturbance of the solvent structure. The IR spectra show about 10% non H-bonded OH groups in water at room T. That means in water there are about 11 mole/liter active non self-associated OH groups. The chemical activity of non H-bonded OH groups is demonstrated at the critical T of water. There nearly all OH groups are non bonded. Water at T_c is strongly corrosive with saphire as well. Beerbower *et al.*[81] have introduced the solubility parameter $\delta = (\Delta U/V)^{1/2}$, ($\Delta U$: energy of evaporation and V: mole volume, $\Delta U/V$ is a measure of the cohesive energy) and a hydrogen-bonding-index (HBI). Both values δ and HBI of water are the largest of all solvents. This demonstrates the excellent solubility properties of water.

Spectra of NH_3 in water show a decrease of intensity in the frequency region (1.4–1.45 μ) of the non H-bonded OH groups of water and an increase in the region of the H-bonds[82] (1.47–1.65 μ). The NH vibration of NH_3 in H_2O shows similar frequencies as are to be found in dilute solutions NH_3/CCl_4[82]. This observation agrees with the model that NH_3 prefers configurations at the surface of the H-bond water aggregates with H-bonds OH· · ·NH. The OH groups of alcohols have similar H-bond properties as water. In H_2O/CH_3OH mixtures with an excess of methanol the IR H-bond band of water is similar to pure water. [λ_{max} (20 °C) = 1.935 μ]. This indicates that the $H-O-H\cdots\overset{H}{\underset{\backslash}{O}}-CH_3$ bonds have similar H-bond interactions like $H-OH\cdots\overset{H}{\underset{\backslash}{O}}-H$ (Fig. 12). During dilution of such mixtures with CCl_4 there appears a sharp IR band of non H-bonded OH groups of water at the same wavelength as in pure water at high T ($T \approx 400$ °C)[49] (Fig. 12). One can assume from this band maximum that in diluted mixtures water/methanol there are many water molecules with both OH groups non H-bonded. The cause of the non H-bonded OH groups in dilution is the small concentration of acceptors.

A comparison of the water spectrum of water-methanol mixtures with the spectrum of liquid water shows that the angle distribution of H-bonds is sharper at $\beta = 0$ and $\beta \approx 180°$. The cyclic structures of water with medium β-values of Fig. 5 seem to be rarer in mixtures than in liquid water. Linear H-bonds $H-O-H\cdots\overset{H}{\underset{\backslash}{O}}-CH_3$ as a consequence of more than 50% non H-bonded lone electron pairs of methanol H-bonds of methanol with water as an acceptor, seem to be preferred. The reduction of the partial molar volume V_1 of water in alcohol mixtures with small water concentration (Fig. 13) may indicate that water in such mixtures lies partially in holes of the ordered methanol structure. In agreement with this conclusion X-ray scattering results indicate an increase of structure in water/alcohol mixtures[83, 84]. The partial molar volume of water V_1 increases by a small alcohol concentration (Fig. 13). These values are calculated from the concentration dependence of density with a computer program[86]. The positive V_1 indicate: the content of the ordered areas of H-bonds

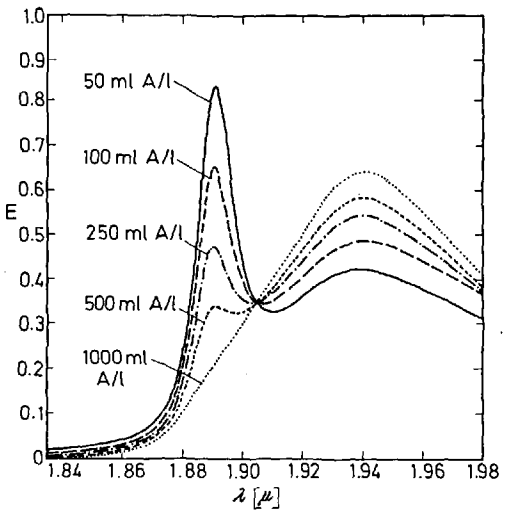

Fig. 12. Combination band of water in water methanol mixtures (A = 20 H_2O/liter methanol) diluted with CCl_4. Parameter ml of A per liter CCl_4. 20 °C, d = 10 cm, methanol spectrum substracted by compensation[82]

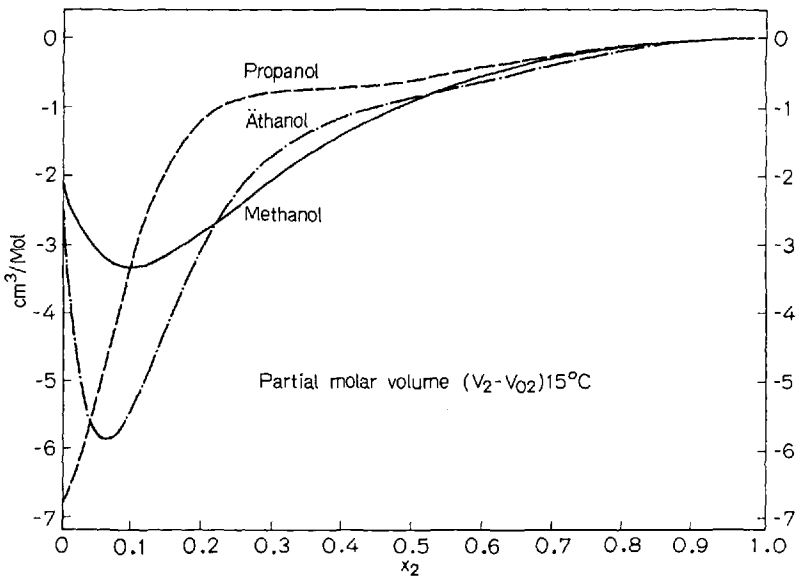

Fig. 13. Partial molar volume of alcohols V_2 as a function of the alcohol mol fraction x_2 in water-alcohol mixtures at 15 °C. V_{02}: mol volume of the pure alcohols. (Calculated by densities values of D'Ans-Lax[85])

with a larger volume increases by water-alcohol bonds at the expense of the non H-bonded disordered areas. The partial molar volume V_2 of alcohols is smaller than the mole volume of alcohols (Fig. 13 and [86]) this indicates that the hydrophobic groups of alcohols may be partially arranged in the holes of the ordered water structure. In correspondence with this hypothesis the excess partial molar volume $V_2 - V_{20}$ of alcohols in water gets larger negative values with increasing size of the alkyl groups groups[86]. For $x_2 < 0.1$ there are negative values of the partial molal enthalpies of ethanol until -2 kcal/mole[84]. The entropies of soluted alcohols are also negative like apolar solutes[87]. Otherwise the linear relationship between entropy and enthalpy of alkanes and alcohols in aqueous solutions are only shifted parallel at a constant factor[87]. X-ray scattering experiments on H_2O/ethanol mixtures varify also a higher order near 0.1 mole percent ethanol[83]. In this concentration region a continuation of the water aggregates by H-bonds water-alcohol may occur. At 25 °C there are minima of the excess partial molal volume at the following molefractions x_2 of the solute: tert.-BuOH 0.028; Et_2NH 0.042; $EtNH_2$ 0.042; Dioxan 0.047; EtOH 0.08; $MeNH_2$ 0.14[86]. Extreme values of properties in mixtures at $x_2 \approx 0.1$ are common. This reminds one of the experience of crystallography showing that foreign particles can be included in a crystal-lattice up to about 10% if the radius of the guest molecule does not differ to much from the lattice dimensions.

Kochnev ([19] Vol. III, p. 7) has found a frequency shift of the $\nu_2 + \nu_3$ combination band with added concentrations of x_2 (methanol) = 0.15; x_2 (ethanol) = 0.1; x_2 (n-propanol) = 0.08; x_2 (t-butanol) = 0.07; x_2 (sec-butanol) = 0.04; x_2 (n-butanol) = 0.0015. Kochnev ([19] Vol. III, p. 7) has found a frequency shift to smaller values of the $\nu_1 + \nu_3$ combination IR band, up to a limit of added concentrations of:

n-butanol $x_2 = 0.02$; sec-butanol $x_2 = 0.04$; t-butanol $x_2 = 0.08$;
n-propanol $x_2 = 0.09$; ethanol $x_2 = 0.1$; methanol $x_2 = 0.12$.

Sidorova, Khaloimov and Zukovskij ([19] Vol. 3, p. 1) gave similar observations for water with different acceptors with the following concentrations of the break of:
acetamide $x_2 \approx 3 \cdot 10^{-4}$; chloral hydrate $x_2 \approx 10^{-3}$; hexanol $x_2 \approx 10^{-3}$;
urethane $x_2 \approx 10^{-3}$; ethyl ether $x_2 \approx 3 \cdot 10^{-2}$; formamide $x_2 \approx 5 \cdot 10^{-2}$.

Both papers would agree with the working hypothesis that solved hydrophilic molecules are bound with low concentrations at the surfaces of the H-bond systems in liquid water and reduce the content of non H-bonded OH groups of water.

Vuks, Lisnyanskij and Shurupova ([19] Vol. II, p. 79) have observed maxima of the isotropic light scattering in water propanol-mixtures. They found sharp maxima at $x_2 = 0.15$ (22 °C).

In mixtures water and solvents with lone pair electrons, the structure depends on the base strength of the lone pair electrons in the series given on page 9. For example the spectra of water-dioxan (Fig. 14) show a weaker frequency shift in comparison with water-alcohol mixtures (Fig. 12) – that means weaker H-bonds – of the H-bond band of water (1.92 μ instead 1.94 μ). The wavelength 1.896 μ in Fig. 14 of the non H-bonded OH band instead 1.89 μ in water/methanol (Fig. 12) corresponds with a non H-bonded water OH group whose second OH is H-bonded (Compare the free OH band in liquid water 200 °C $< T <$ 350° in Fig. 12[49]).

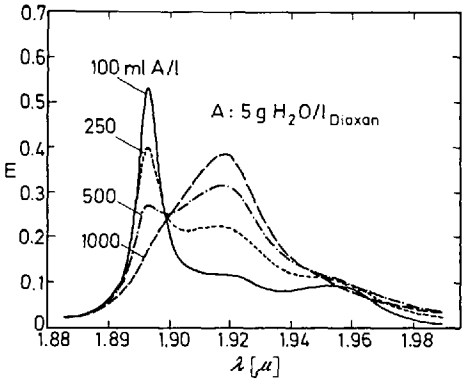

Fig. 14. IR combination band of water in dioxan mixtures diluted with CCl_4 at 20 °C. Numbers on the curves give the amount of the mixture A per liter CCl_4, cd = 500 (g l^{-1} cm)[82]

The properties of water in mixtures with hydrophilic organic molecules will depend on the base strength of the acceptor, on the acidity of protons of organic molecules and on the concentration-ratios. We have to take note that strong interactions between hydrophilic molecules can prevent miscibility with water. Preference of special H-bond orientations can induce inmiscibility. Mucic acid with 6 OH groups is insoluble in water but saccharic acid with the same groups is soluble[84]. Scyllo-inositol with 6 OH groups is insoluble, but myo-inositol is soluble[84]. In both cases the only differences are in the conformation.

The H-bond model of hydrophilic solutes at the surfaces of the large water aggregates becomes more probable by a reduction of mobility of water molecules by solutes. With NMR and electron paramagnetic resonance technique can be shown[84] that the diffusion motions of water show a general slowing down by tetrahydrofuran, tert-butanol or aceton additions[88–92]. From the point of view of the reduction of the mobility of water in mixtures a stability effect on the water structure was claimed[92]. This stability effect decreases with T[92]. The observed rotation degree of freedom of solutes[84] tallies with our model too.

The low viscosity of water with an activation energy of about 4,8 kcal/mole at 0 °C and 2.8 kcal/mole at 100 °C (Fig. 15) agrees with the assumption that the viscosity of water depends mainly on a rearrangement of the water structure, starting at the cluster surfaces with water molecules of one H-bond or at areas with angle unfavoured H-bonds. The activation energy of water diffusion is given by NMR data at 4.6 kcal/mole[92] and by tracer data at 4.8 kcal/mole at room T[93]. The activation energy of NMR proton relaxation time T_1 of water is given at 5.3 kcal/mole[92]. These energies are in the size of order to solve one H-bond of water. In the cluster model there are molecules with one H-bond at the cluster surfaces only. In this model solutes would partially harden the mobility of the boundaries between different water aggregate systems. The property of water to build gels with very small additions can be included in this model too. For example by small amounts of the dyestuff pseudoisocyanin water becomes a gel. Together with the gel formation a sharp absorption band of large associates at 580 mμ appears (Fig. 16)[94]. In the gel phase a sharp association band of the dye appears[95] (1 dye per 2000 water molecules). This asso-

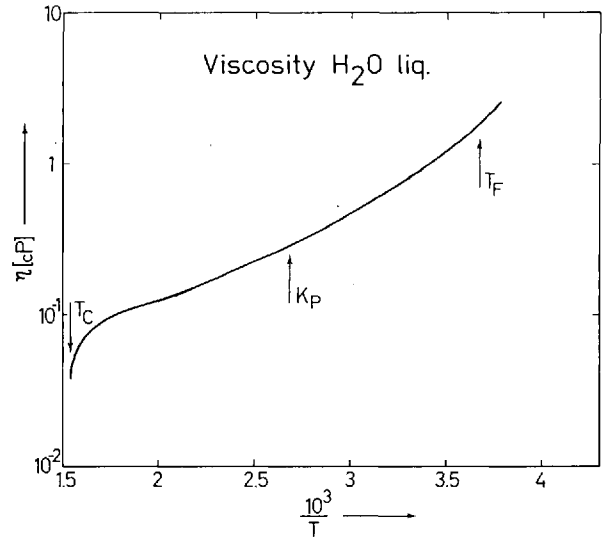

Fig. 15. Viscosity of liquid water under saturation conditions as a function of $1/T$ (plot of data given in[97])

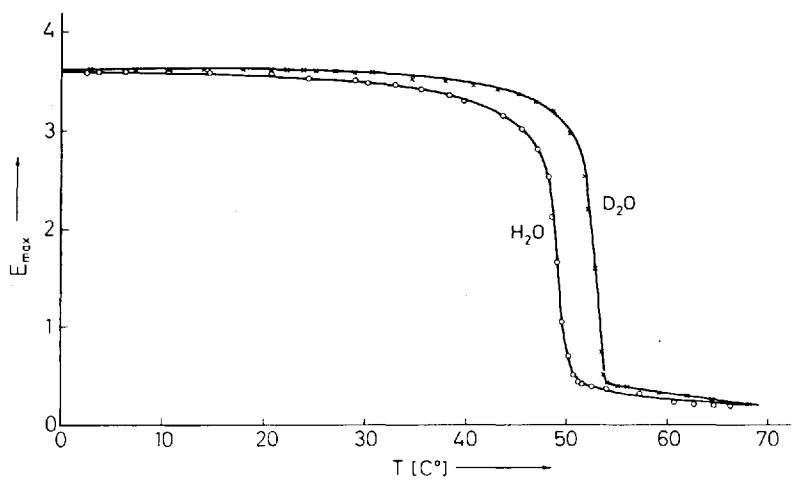

Fig. 16. The gel formation of pseudo-isocyanine-chloride in water (7,5 g/l) determined by the optical density E_{max} of the association band at 570 mμ. The gel formation in D_2O changes about 3.8 °C, the melting point difference D_2O–H_2O; D = 0.02 cm[94]

ciation band disappears during drying these samples and appears with adding small amounts to water[96] depending on the pH-value[94]. This is a good example for special properties of hydrates. It is known too that small amounts of silicon (1 SiO_2 : 330 H_2O) change the water structure to a gel[97]. This is also the cause of the known

story of the so-called polywater[98, 99]. It is built in quartz capillaries by small amount of impurities[98]. Polywater consists of a gel formation by impurities. –

There is a direct proportionality between the NMR correlation time – as measure of the mobility – and the IR frequency shift of the OH-bond of water – as measure of the H-bond interaction and order effects – in organic solvents[100].

The properties of ternary systems depend on the affinity of the three components. For example HCl or H_2SO_4 have a larger affinity to the amino-endgroups of polyamide than to water. Therefore, HCl at medium pH has partition coefficients k between 6-polyamide fibres and water of about 1000[101]. This gives a very slow extraction process for HCl from polyamide. If n are the number of extraction equilibrium steps with water the percentage of solute inside the polyamide fibre is given by a function[102] with the partition numbers: $G = k\ V_1/V_2$ as parameter. If m_{Fo} is the amount of dye or acid drawn up in a one step dyeing process and if m_F is the amount of dye or acid inside the fibre after n replacements of new solvent outside the fibre: for $G = 3000$ $m_{Fo}/m_F = f(n)$ is given in Table 3[101]:

Table 3.

n	$100\ m_{Fo}/m_F$
1	99.96%
10	99.6 %
100	96.7%
1000	71.6%

This wash-fastness mechanism of acid dyes on polyamide fibres may be a model for the uptake of nutritive substances, of drugs and so on by the organism or the uptake of K^+ by complexes in cells too.

c) Non-electrolyte Solutions with Hydrophobic Solutes

The high interaction energy of water interprets the so called hydrophobic interaction too. To solve larger hydrophobic molecules or groups in water it would be necessary to disrupt H-bonds between the water molecules. The intermolecular forces between water and hydrophobic molecules or hydrophobic groups of organic molecules would be much lower than the H-bond interaction between water molecules. Therefore, a cluster-formation of organic molecules or organic groups in aqueous systems can be energetically preferred, this effect is called the hydrophobic interaction. This nomenclature is not the best one because it induces the thought that there would be special hydrophobic forces. We have to stress that the cause of hydrophobic interaction is the strong H-bond interaction of water. Tanford[103] pointed out that the word "hydrophobic" is a misnomer by a different point of view. It gives this impression if the organic molecules are averse to water, but the facts would be better described by saying that water is averse to the organic molecules. Franks[86] and Ives[104] use the term "hydrophobic hydration". Lauffer[105] who distrusts the nomenclature hydrophobic bond too "because there need not be any forces at all between the protein surface", prefers the concept "entropic union". We could expect that smaller molecules would solve in the fissure plains of orientation defects between the big water associate systems. But from the point of view of high density the cohesive energy

should be still high in the areas of non H-bonded OH groups. The solubility of small hydrophobic molecules in water induces a positive change of the entropy. The classic example for this effect is the transfer of one mole of methane from an aqueous solution to ether. It is $\Delta F = -3.3$ kcal/mole; $\Delta H = 2.4$ kcal/mole and $\Delta S = 19$ cal/deg[105, 106].

Table 4 gives the dependence of these values from the size of the hydrocarbon[107]:

Table 4. Free energy, energy, and entropy of migration of hydrocarbon from water at 25° to nonpolar environment

Compound	ΔF_{tr}, kcal/mole	ΔH_{tr}, kcal/mole	ΔS_{tr}, e.u.
CH_4	-3.0	+2.6	+18
C_2H_0	-3.8	+2.0	+19
C_3H_8	-5.0	+1.8	+23
C_4H_{10}	-5.8	+1.0	+23
C_6H_6	-4.3	$0 \sim -0.6$	+14
$C_6H_5 - CH_3$	-5.0	$0 \sim -0.6$	+16

The free energy change prefers the mixture methane/ether in contradiction to the energy difference. This common entropy effect has caused the model that water becomes more ordered by hydrophobic solutes. Frank and Evans[106, 108] spoke about an "ice-berg"-forming around hydrophobic solutes. This term has caused some confusion. There are no indications that the H-bonds – the cause of order in water – increase by hydrophobic solutes[109]. There is no experimental evidence and no theoretical reason for an increase of H-bonds, therefore we have proposed[110] to see if the entropy effect in such mixtures could be caused by the forming of five-membered ring structures in aqueous solutions.

Water forms five membered rings in so-called gas-hydrates or clathrates[111–113]. Small molecules like CH_4 or Cl_2 build under pressure solid clathrates in water with melting points higher than 0 °C. In clathrates 12 plain five membered rings of 46 water molecules are arranged in a pentagondodecaeder. The pentagondodecaeder includes a hole (Fig. 17). In molecular models the hole has the size of about a benzene molecule. This structure stands out by the fact that all "hydrophilic" groups of a water molecule, the protons or the lone pair electrons, turn its back upon the included hole. The water molecules turn its most hydrophobic face toward the guest molecule which is fixed in the centre of the hole. The H-bond energy in the five membered rings with $\beta \approx 3°$ is a little less than in the ice lattice (see Fig. 4). This energy loss has to be compensated by the dispersion forces of the guest molecules with the surrounded water molecules. In the example given above the dispersion energy of methane/water may be larger than in methan/ether. The dispersion energy between the guest molecule and the water clathrate molecule is fairly high because the guests have a large co-ordination number, and its dispersion energies can be estimated summing up the effects of all its neighbours. The enthalpy ΔH for transfer 1 mole methane from clathrate to gas and ice is 4.5 kcal/mole[84]. For our model of the liquid hydrophobic mixtures with water we can compare this with the 2.2 kcal/mole for transfer of 1 mole methane from aqueous solutions to ether solution. Over-

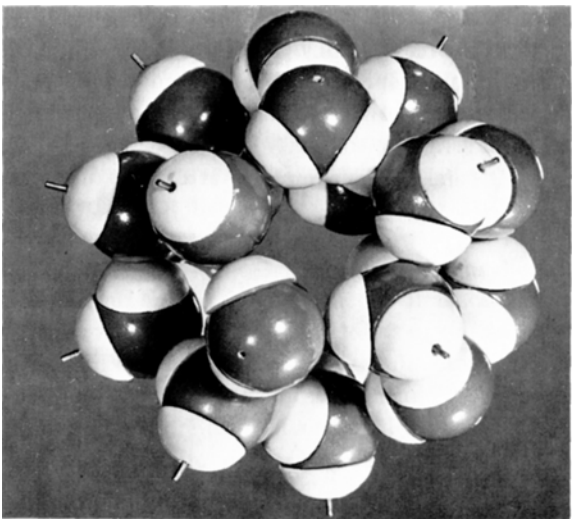

Fig. 17. Model of the clathrate structure of the water in clathrates I. The centre of the hole is occupied by hydrophobic guest molecules in gas-hydrates (model of the iceberg formation in aqueous solution?)

tone spectra of SO_2-clathrates establish the smaller interaction of water in clathrates in comparison to ice[82]. NMR chemical shift measurements of aqueous solutions of hydracarbons show an upfield shift which usually correspond to a decrease in hydrogen bonding[114]. This would agree with our model of these systems.

The plain five membered rings of clathrates may have a lesser degree of freedom as the non-plain six membered ring of the normal ice structure. We can expect that the entropy of this structure is smaller than in the ice lattice. This model could interpret the entropy effect of aqueous mixtures without structure increase.

Already Eley has supposed that the negative entropy of mixing is related to the cavity formation process itself[115]. Assumptions that the negative entropy effect may be mainly caused by a restriction of the mobility of the chains of the organic solutes can be cancelled, because inert gases have the negative entropy effect in aqueous solutions too[87], and relaxation measurements have shown that tetrahydrofuran in water rotates as freely in aqueous solutions as in the pure liquid[87]. Normal clathrates I with pentagondodecaeder structure are known with guest molecules up to 5 Å in size[113]. The so called structure II of clathrates is formed by a unit cell of 136 water molecules which consists of a mixture of water five membered and six membered rings[113]. Clathrates with structure II are known with guest molecules with diameters of 5.6–6.6 Å[113]. One example is propylene oxide[113]. Aqueous mixtures with larger hydrophobic molecules can be built by neighbouring of some pentagondodecaeder or similar structures which form larger holes or by forming micelles of the hydrophobic groups as in soaps. Such micelle building is called „hydrophobic bond". But one has to stress that the main cause of this micelle forming is the possibility to solve hydrophobic groups with a minimum of opening H-bonds in water.

The solubility of small molecules like rare gases or $CH_4 \cdots C_4H_{10}$ in water decreases with T[84, 116] but increases with T in cyclohexane. This indicates too that the solubility mechanism in water depends on a special ordered structure forming of water. Benzene and derivatives show a minimum of the T-dependence of the solubility in water. In homologous series the solubility in water decreases with increasing radius of the solute[87] (Fig. 18) and becomes very low at a radius of more than 6 Å. This

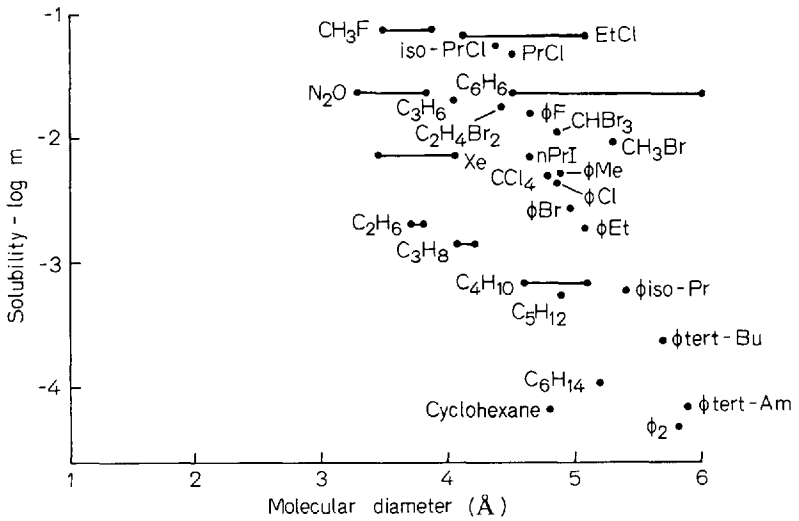

Fig. 18. Logarithm of the solubility of organic molecules in water at 25 °C as a function of the molecular diameter in Å. (Figure from[87])

agrees with the hypothesis of a disturbed clathrate-like arrangement of water around apolar solutes. The hole formation by clathrate-like arrangements in liquid water and the large co-ordination number of a guest molecule inside such holes may be favoured in comparison to a solubility mechanism between orientation defects and an increase of the orientation defect content. The dynamics of the mobility of orientation defects is to be taken in account too. The larger solubility of benzene compared with cyclohexane may be influenced by the weak acceptor function of π electrons for H-bonds[117].

Contradictory to the radius dependence of apolar solutes the solubility of the rare gases in water increases with the radius[87]. But this may be an effect of the increasing dispersion forces with the molecular weight of the rare gases.

There are some other arguments including molecular dynamics calculations[46, 84] for the clathrate-like arrangements of water in solutions of apolar solutes[118–121]. When methane is transferred from water to hexane an increase of volume of 22.7 ml/mole is observed[105]. This large volume effect can be easily understood on the assumption that methane in aqueous solutions is arranged in holes of the water structure. The negative excess partial molal volumes of solutes with hydrophobic groups in water indicate that holes in the water structure are participating in the solubility mechanism. Glew[122] has interpreted the NMR chemical shifts in dilute aqueous

solutions by clathrate-like structures[123]. He points out that the observed extreme properties of aqueous mixtures correspond to the clathrate stoichiometry[84, 113] Amines form semiclathrates with water[113]. In this case clathrate-like structure formed but the N-atoms are acceptors for water H-bonds. There is, for example, a hexahydrate of hexamethylentatramine[113]. In this case three of the four-N-atoms are H-bond acceptors. In the semiclathrates deviations of the H-bond angle $\beta = 0$ occur[113]. NMR spectra, IR or dielectric methods show a high rotational mobility of the guest molecules in clathrates[113].

A direct relation between the biologic applications and the discussed clathrate model of hydrophobic mixtures is Pauling's theory of anaestesia. He has suggested that anaestesia may be attributed to the formations of clathrate microcrystals in the brain[124–127]. The basis of Pauling's theory is the direct proportionality between the log of the minimum pressure to anaesthetize a goldfish with one chemical and the log of the minimum pressure to form a gas-hydrate at 0 °C with it. Values are given of 0.04–0.08 moles per liter of brain lipids for the concentrations of anaesthetics[128].

The so called hydrophobic bond has a fundamental importance for biology. It is the cause that living organism do not solve in water although they consist mainly of water.

d) Mixed Aqueous Systems Hydrophilic-Hydrophobic

In a large group of aqueous systems the solutes have both hydrophilic and hydrophobic groups. Polyethylenoxid-derivatives with hydrophobic endgroups are a good model to study such systems. They form micelles in aqueous solutions[129, 130]. In a very diluted solution monomers dominate and σ decreases proportional to the concentration c. Starting at a more or less critical concentration C_c micelles are formed. Above C_c the monomer concentration is nearly constant and the surface tension gets constant (Fig. 19). The cause of a "critical concentration" is the association

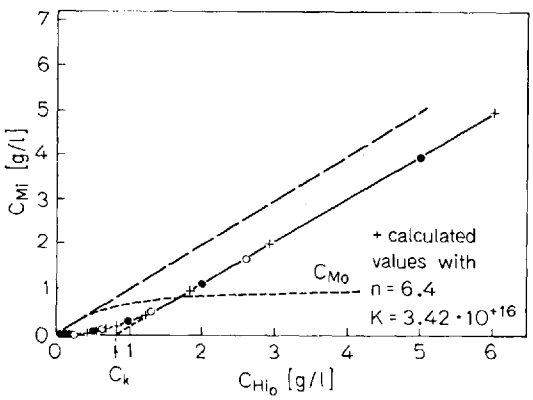

Fig. 19. The micelle concentration of PIOP-25 in water (PO_4-puffer pH = 8) at 20 °C as function of the concentration by weight. The short dotted curve gives the monomer concentration Mo, the long dotted curve the 45° slope[72]

equilibrium between many molecules[129, 130], in this example six[129]. The micelles can be observed spectroscopically with iso-C_8H_{17}-$(O-CH_2-CH_2)_n$-OH. Above C_c a peak of the phenylband at 280 mμ appears[130] (Fig. 20). This peak is observed in

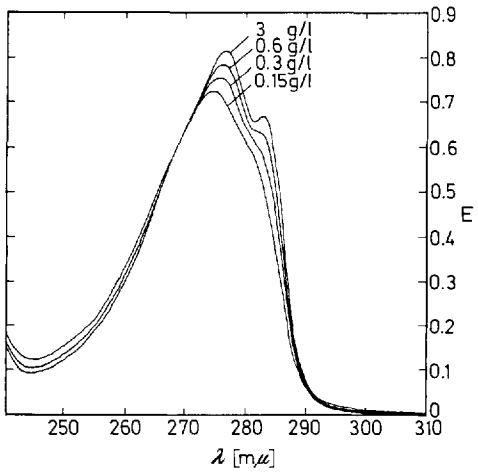

Fig. 20. The UV phenyl-absorption band of PIOP-7 as function of the concentration in water at 23 °C. At 283 mμ appears the micelle peak, which can be observed of PIOP-7 Mo monomers in organic solvents. This peak indicates the organic environment of the phenyl rings in micelles[72, 129]

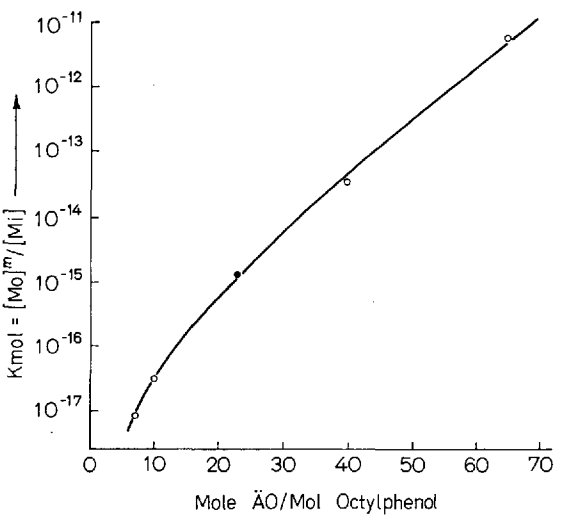

Fig. 21. The micelle formation equilibrium constant $K = \frac{Mo^6}{Mi}$ of PIOP-n as function of the number n of ethyleneoxyde groups at 20 °C in water pH = 8[72]

solutions of hydrophobic organic solutes as well and indicate a non-aqueous environment. The micelle equilibrium of

$$C_8H_{17}-\langle\bigcirc\rangle-(O-CH_2-CH_2-)_nOH \text{ (abbr. PIOP-}n\text{)}$$

depends on the hydrophilic-hydrophobic balance HHB of the hydrophilic and the hydrophobic part of these molecules. Figure 21 gives the spectroscopic determined equilibrium constants K_{mol} of micelle building of PIOP-n as function of n[130]. The HHB is sensitive to salt additions. An addition of 0.5 mol Na_2CO_3 to PIOP-40 reduces at 20 °C K_{mol} like a reduction $n = 40$ to $n = 10$. At $C_{salt} = 0.5$ mol/l the series of salt-influence on K_{mol} is:

$$Na_2CO_3 > Na_2HPO_4 > Na_2SO_4 > (NH_4)_2SO_4 > NaOH > NaHCO_3 > BaCl_2 > NaCl > LiCl.$$

The dependence of K_{mol} on the salt concentration varies a little with the salt's type. Therefore, this salt series varies a little with C_{salt}. The influence of the different ions to K corresponds to the Hofmeister ion-series or the ion-series got by the influence of the water structure[31, 72].

Structure maker ions increase the concentration of micelles and reduce the concentration of monomers. In a very rough model one can assume: the fixed hydration sphere around the ions cannot solve the ethylenoxide products and increase its concentrations in the rest bulk water phase (salt-out effect). In this model one can estimate the size of the hydration sphere of the ions. The hydration numbers gained by this method are surprisingly large[31]. They start at 200 water molecules per ion pair at 0.1 mole solutions of ions and decrease about 20 at 1 mole solutions[31, 72, 130]. It is useful to call these numbers secondary hydration numbers. They indicate a long range disturbation of the water structure by the ions. With different other methods one obtains similar numbers, for example, by the ion effects on partition coefficients of organic solutes in water/cyclohexane[31] or by ion effects on the solubility in gases[31, 131]. The secondary hydration numbers have similar sizes like the spectroscopic determined changes of the water aggregate numbers of water[132]. For example, from the partition coefficient of p-cresol between cyclohexan/water we gained[133] secondary hydration numbers SHN of Na_2SO_4 Fig. 22 and of NaCl Fig. 23. In agreement with the IR spectroscopic results the SHN values of sulfate decreases with T (reduction of the salt-out effect by T) and the SHN values of NaCl depend less on T. These experiments with ethylenoxid derivates indicate that we can differ between a primary bihydrate and a secondary hydrate of about 20 water per ether oxygen atom. The existence of a weak bonded secondary hydrate may be indicated by viscosity measurements at low T too. In this region a complete solubility exists. But the viscosity shows an increase above a concentration of PIOP-9 of 200 g/l, that means in such solutions with an enlarged viscosity the total water amount is lower than 15 water per oxygen atom[31]. The high viscosity may indicate a fixation of the secondary hydrate structure. In a concentration region of 15 to 4 water per oxygen atom the activation energy of the viscosity increases with increasing water content. This may indicate a stronger interaction water-solute with increasing water content.

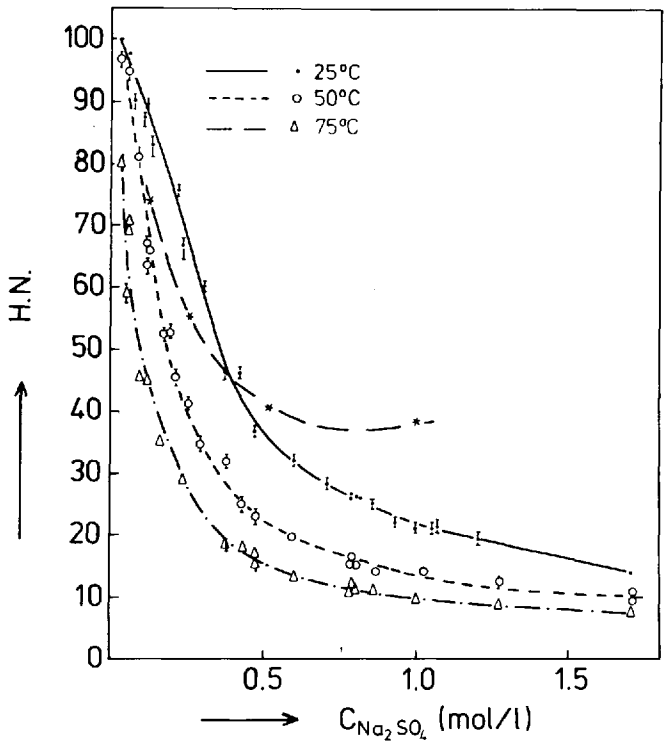

Fig. 22. Appearent hydration number (HN) of Na_2SO_4 determined from the partition coefficient of p-cresol in cyclohexane/water[133]

A model of the micelles of the polyethylenoxide derivates would contain a nucleus of the hydrophobic iso-octylphenyl groups and the water "soluble" polyethylenoxide chains. The micelle nucleus can solve other organic molecules for example dyestuffs and induce a solubility effect of solutes by a hydrophobic bond effect. These complex compounds dyestuff micelle-nucleus have a buffer effect on the dyestuff and play an important role in textile dyeing processes[129, 134].

Other association effects by similar hydrophobic effects are known. For example, a lot of dyestuffs aggregate only in water[135, 136]. This aggregation of dyes can happen too with dye-ions against the Coulomb repulsion forces[135]. We found one example bromphenolblue: it aggregates even stronger in the charged dissociated form than in the undissociated ones. Strong ordered hydration spheres around the ion-group seem to give an isolation effect on the charges[129].

e) Entropy Driven Processes

The aqueous solutions of p-iso-octyl-phenyle-$(-O-CH_2-CH_2-)_nOH$ show a demixing phenomenon at high T. Above a turbidity point T_T two phases appear (Fig. 8). The turbidity point T_T is a measure of the HHB-balance too. Fig. 24 shows T_T as function of the concentration C_{Hi} of PIOP-n. With the length n of the ethylenoxid

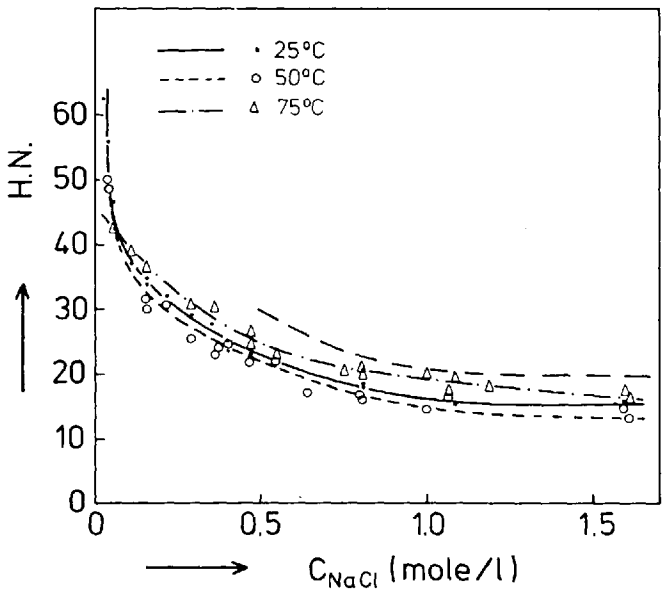

Fig. 23. Appearent hydration number (HN) of NaCl determined from the partition coefficient of p-cresole in cyclohexane/water[133]

chain the hydrophilic properties increase. Until concentrations $C_{Hi} \lesssim 100$ g/l T_T change only little with C_{Hi}. This indicates that T_T is not influenced by an equilibrium of PIOP-*n* but more by the water structure. This effect serves as a model for a lot of biochemical observations: in which the structured state is favoured by an increase of T[105] (polymerisation of tobacco mosaic protein; polymerisation of sickle-cell anaemia hemoglobin; division of fertilized eggs, collagen fibres, formation of pseudopodia etc.[105]). Manly, these effects are endothermic, Lauffer calls it entropy-driven processes[105]. He assumes that the entropy of water increases and the hydrophobic interaction of organic molecules favoured this structure preference at high T. For example, tropocollagen obtained by extracting from rabbit skin with neutral salt solutions, is soluble at 4 °C and forms collagen fibres at 37 °C[105, 137].

The organic phase of the ethylenoxide products with higher viscosity above T_T is a simple model of the formation of gel structures of proteins at higher T. It can be shown[105] that during polymerisation of tobacco mosaic virus, protein water is released and the total volume increases similar to the volume increase during the transfer of methan from water to hexan. Lauffer[105] assumes that the driving force of entropy driven processes in biology is the entropy increase of the released water. The amount of released water is small: 0.027 g water per gram protein or 26 water molecules per chemical repeat unit of protein, about 1/10 of the total "bound" water available. The influence of ions on the HHB of PIOP-9, determined by T_T was given in Fig. 8.

During decompose the long range water structure changes can be studied by the polyethyleneoxide derivatives too. At the turbidity point T_T the organic phase con-

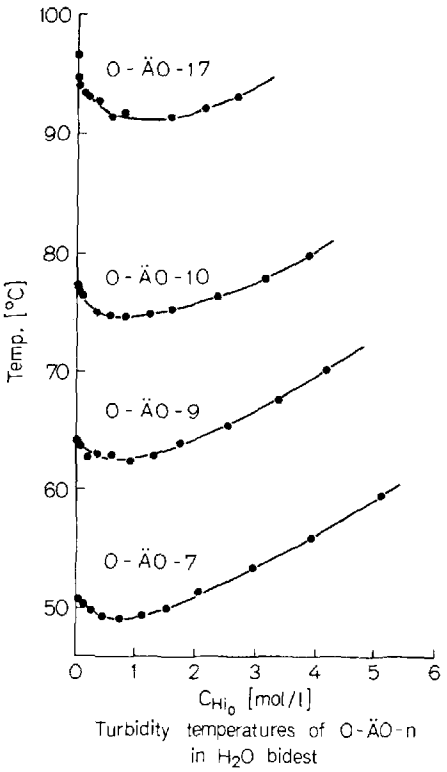

Fig. 24. The turbidity point T_K of aqueous solutions of PIOP-*n* as a function concentration of ether oxygen of PIOP-*n* and the ethyleneoxide-content *n* as parameter[224)]

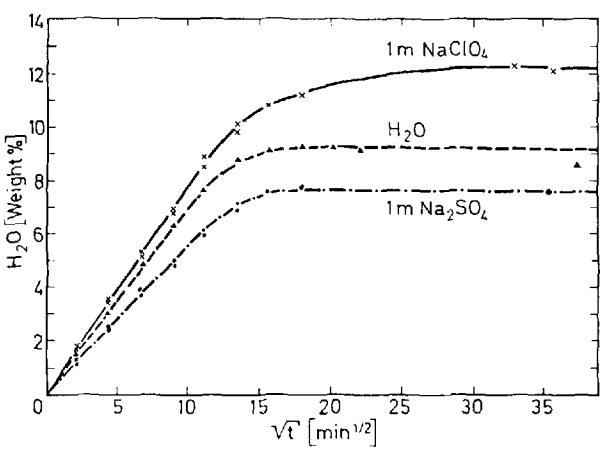

Fig. 25. The water content of 6-polyamid (1 mm thick) as a function of the time *t* and salt additions[113)]

tains about 20 water molecules per ether oxygen atom. The water content of the organic phase after demixing can be reduced by an inrease of T or by added structure breaker ions in the water phase[31]. This reduction of the water content by structure maker ions seems to be a common effect. We could observe that the water equilibrium content of polyamide fibres is reduced by structure maker ions such as Na_2SO_4 [31] or increased by adding structure breaker ions such as $NaClO_4$ (Fig. 25). A dehydration of the human skin may occur in salt-containing thermal baths too.

The $NaClO_4$ additions increase also the water content of the organic phases above T_T of $C_8H_{17}-\langle\bigcirc\rangle-(O-CH_2CH_2)_9OH$[31]. The water content of the organic phase above T_T of PIOP-9 as function of different ions is given in Table 5. The concentrations in the one phase below T_T has been: PIOP-9 100 g/l and salts 0.5 mol/l.

Table 5. Water content in mole H_2O/ethylenoxid-unit at 80° of PIOP-9

NaSCN	18
$NaClO_4$	14.3
NaJ	11.5
Without salt additions	4.8
$NaNO_3$	4.4
NaCl	4
$ZnCl_2$	4
NaBr	3.9
$CaCl_2$	3.8
LiCl	3.7
NaOH	3.3
KCl	3.2
$MgCl_2$	3
NH_4Cl	3
$AlCl_3$	2.8
NaF	2.6
Na_2CO_3	2.2
$NaHCO_3$	2.2
$CuSO_4$	2
$MgSO_4$	1.8
$ZnSO_4$	1.7
Na_2SO_4	1.6

Table 5 gives indications of the influence of the Hofmeister ion series – as measure of the water structure – on the water content of organic phases in aqueous two phase systems. The polyamide experiments (Fig. 25)[127, 138] assume that this is a common effect and may happen with biopolymers too (see Chapter III). The organic phases of PIOP-9 contain ions also. If one heats the organic phases the released water has an ion content similar to the original ion concentration before demixing in two phases. Another interesting effect has been that both factors heating or adding structure-making ions can reduce the water content of the organic phase only up to 2 water per oxygen atom if the starting PIOP concentrations are not too high. This bihydrate has extreme properties[31]: a viscosity maximum, a maximum of the veloc-

ity of sound and a special X-ray structure. These properties indicate a special bihydrate with more fixed bonded water. New spectroscopic IR studies established that this bihydrate is accompanied by special water structures[139]. Such organic primary hydrates may depend on special arrangements with H-bond angles $\beta = 0$[31]. In the case of ethylenoxid-derivatives special helix structures were found[140].

In biopolymers hydrates may enforce special conformations[141]. For example, the harmone oxytocine of the hypophysis conforms with all hydrophobic groups on one side[127] (Fig. 26) and the hydrophilic groups on the other[127] (Fig. 27). Similarily the A-chain and the B-chain of insulin can be arranged to form a disc with a

Fig. 26. A model of the hormone oxytocin, conformation with a concentration of the hydrophobic groups on one side[127]

Fig. 27. The front side of the model of fig. 26 with a concentration of the hydrophilic amide groups to the front[127]

concentration of the hydrophobic groups on one side and the hydrophilic on the other[127, 141]. Warner[141] has found similar possibilities with the antibiotics gramicidine-S. These configurations of insulin or similar structure of tobacco-mosaic virus show six membered rings of oxygen atoms with 0–0 distances of 4.8 Å. This distance corresponds to the 0–0 distance in ice from one 0 to the next but one. Warner[141] assumes that this distance induces preferred hydrates with ice-like structures (angle $\beta = 0$). Figure 28 gives a model of the ice lattice (below) and a peptide chain (above).

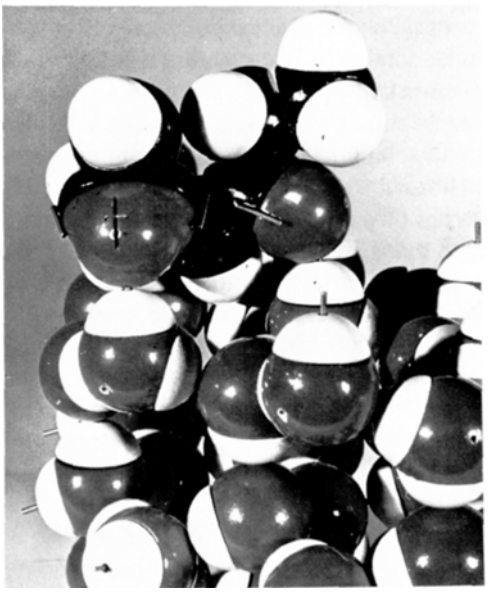

Fig. 28. Below: a model of the ice lattice. Above: a peptide group in a conformation with H-bond angles $\beta = 0$ to the water molecules[127]

The peptide group can be fixed at the ice lattice with $\beta = 0$[127]. Ice-like distances of the H-bond acceptors are in different molecules: for example: Biotin, Thymine, Triglyceride, 1,4-Quinone etc.[141]. Hechter gave a model for biologic membranes too[142]. In the membrane surface Hechter assumes a water double hydrate lamella of 4.9 Å thickness in which K^+ could be placed, but Na^+ would be too big.

The differences in solubility between mucic acid/saccharic acid or scyllo-inositol/myo-inositol may be influenced too by different similarities of the oxygen spacing with the lattice-like ordered system of water molecules[87]. The hydroxyl-orientation plays also an important role with different properties of 2-/β-mannose or 2-/β-glucose in aqueous solutions[87, 143]. The specific hydration by relative positions of the polar groups of the solute and its compatibility with the distances of the water association system can be an important factor in the interaction hydrophilic-groups water[144]. In some cases one specific conformation of organic compounds or enzymes may be stabilized by the water structure. This may be important in some biologic systems.

Jaenicke and Lauffer[105, 145, 146] have found changes in the optical rotatory dispersion when the tobacco mosaic virus polymerizes. Lauffer[105] interprets this observation as a change of the protein conformation effected by the different structures during exposure on all sides to water or in contact with other protein molecules. The large water contents of the organic PIOP-*n* phases indicate that biopolymers may contain hydrates too. This possibility is neglected by some authors. The positions of polar groups of the solute in relation to the size of the non-polar group is a further parameter which induces the big scale of aqueous-mixtures properties.

The ion content of the organic phase of ethylenoxid-products indicate that under saturation conditions there are some water molecules whose properties are not too different from normal water. Polypropyleneoxide products which contain much less water, release under similar conditions water with a reduced ion content[147]. From this experience one gets a working hypothesis for the mechanism of semipermeable membranes. The membranes should have some "secondary" hydrate shell with movable water but by reason of solubility or steric effects, not too much "secondary hydrate water" to avoid normal water with common solubility properties.

The different properties of water with different bonds to organic molecules can be demonstrated also with dye diffusion measurements in polyamide fibres[148, 149]. Acid dyes show a remarkable decrease of diffusion velocity in 6-polyamide if the humidity content is reduced. It is necessary to heat polyamide fibres in air contact at 150 °C to get similar diffusion velocities of acid dyes at 60 °C under water saturation. The diffusion coefficient of water itself in 6-polyamide depends strongly on the water content[148, 149] (Fig. 29). This was determined by a quartz spring balance method.

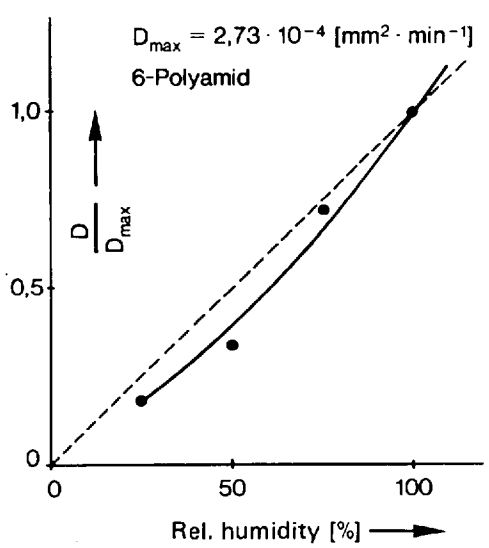

Fig. 29. The water diffusion in 6-polyamide at 80 °C at function of the rel. humidity. The activation energy of D_{max} is 13.7 ± 1.5 kcal/mole for 20 °C; T = 80 °C[149]

This may be important in biochemistry. Under dry climates the surface of plants or animals will dry. Then the water diffusion inside the organism is reduced and the drying process becomes retarded. Spectroscopic measurements of the velocity of water uptake by 6-polyamide samples gave an activation energy of the water diffusion 13.7 ± 1.5 kcal/mole[149]. This indicates the strong interaction energy of small water amounts in polyamide. The activation energy of water in polyamide is much higher than in liquid water.

The important role of different water properties in different structures was given by Hays and Leaf[150]. They have found that the activation energy for water for the transport through pores of 8.4 Å in biologic membranes is 9.8 kcal/mole[150] (corresponding to the heat of vaporisation of liquid water at room T). With injections of vasopressine the radius of pores increases to 40 Å and the activation energy decreases to 4 kcal/mole[150, 151] (approx. the activation energy of diffusion of normal water). Ling, Miller and Achsenfeld[152] also suppose: in frog skin the "pores are much larger than any solute tested and therefore, the selectivity of the membrane must be inherent in the interaction of the solute with the structured water of the skin".

Other examples for stabilisation of structure at higher T in biochemical systems are the gel formed by deoxygenated sickle cell hemolysate[105, 153]. The gel is formed at 38 °C, but liquifies reversibly upon reducing T to 0 °C. Myosin, a protein in the myofibrils of skeletal muscles is soluble in water at 0 °C (pH = 7.4) and not soluble at 23°[105, 154]. – Actin, a component in the filaments of the myofibrils polymerizes at 25 °C and depolymerizes at 0 °C[105, 155]. – The tension of turtle muscles decreases with increasing pressure below 5 °C and increases above 10 °C[105, 156]. Antibody building is favoured more at 25 °C than at 3 °C[157]. Lauffer includes this observation in entropy driven processes too[105].

Poly-L-proline is soluble in water for $-15\,^{\circ}\mathrm{C} < T < +5\,^{\circ}\mathrm{C}$, but precipitates at $T > 25\,^{\circ}\mathrm{C}$[105, 158]. Inoué[159] observed the mitotic cell devision of Chaetopterus pergamentaceous. He could see spindles in a microscope inside the cells, strongly evident at 35 °C. These spindles disappear at $T < 10\,^{\circ}\mathrm{C}$[105, 159].

D_2O blocks mitosis at a concentration higher than 70%[160]. Marsland[160] assumes that deuteration over-stabilises the gel structure. Rowlinson[161] divided aqueous nonelectrolyte solutions into two classes: Those solutes for which $T\,|\Delta S^E| > |\Delta H^E|$ are called "typical aqueous" and those for which $T\,|\Delta S^E| < |\Delta H^E|$ (like nomalous non-aqueous systems) are classified as "typical non-aqueous".

III. Biologic Systems

a) Lipids

Lipids give good examples for mixed systems with hydrophilic and hydrophobic groups and its typical interactions with water. Hauser[162] has given a larger review on this sphere of action. We are reviewing his report and are giving the main results.

Phospholipids consist of two hydrophobic hydrocarbon chains and a polar head group, ionized and/or sugar groups. Above a critical temperature T_c they swell in

contact with water[163]. T_c depends on the melting point of the analogous fatty acid, on salts and pH. Water forms different lyotropic mesophases with phospholipids. There are gel phases of bilayers of the more or less crystallized hydrocarbon chains with water between or coagels: microcrystals and excess water. At higher T there are two types of hexagonal phases, consisting of two-dimensional cylinders. The interior of the cylinder can be lipid hydrocarbon chains with the polar groups at the surface of the cylinder[162]. The different cyclinders are arranged in a hexagonal package with water as connecting element. There are inverse phases with water cylinders hexagonal packed with lipids as connecting element with the polar head groups at the surface of the water cylinders. Above 80 °C phosphatidylcholines can form a phase with finite rods containing the polar groups and water. The rods are forming an interwoven three-dimensional network with a liquid-like hydrocarbon region in between[162]. Shan has demonstrated with NMR technique[164] on microemulsions (1 part hexadecane, 0.4 p. hexanol, 0.2 p. K-oleate in water) that there are different concentration regions with water-spheres/water cylinders and water lamellae. The cylinders have diameters of 1.035 Å and lamellae are 5.30 Å in thickness. This size of order corresponds to the extent of H-bond regions in liquid water.

With different methods one can recognize different hydrates of phospholipids with different properties. Solved in different organic solvents like benzene, xylene etc. egg yolk phosphatidylcholine adsorbed water in an amount of 13.6 water/mol lipid, independently of the used organic solvent[165]. Studying adsorption isotherms of water vapor on lipids Elworthy[166] has found saturation values at 25 °C:

18 mole H_2O/mole phosphatidylcholine or 14 mole water/mole lysophosphatidylcholine. These values depend on T. At 40 °C one mole phosphatidylcholine takes up 20 mole water. With viscosity measurements too a hydration number of 16 mole water/mole egg lysophosphatidylcholine is observed[166]. Elworthy[166] concluded from the slope of the water adsorption different types of hydrates: the first hydration layer of 2.5 mole water/lipid; the second hydration layer at about 6 mole water/mole lipid[162, 166]. With anhydrous 1,2-distearoyl 'L' phosphatidylcholine hygroscopic behaviour is only observed above the T of the liquidcrystal-crystal transition point[167].

Thermal analysis data show that there is a certain amount of unfreezable water in phosphatidylcholin-water systems. Chapman and co-workers[167] conclude that this unfreezable water is bound. Indeed, the amount of unfreezable water is in agreement with hydrated water determined with other methods (egg-phosphatidylcholin 15 mole water[167], egg phosphatidylethanloamine 6 mole water[167], 10 mole water/mole dipalmitoyl phosphatidylcholine[168]). X-ray methods show distinct structures too: egg phosphatidylcholine has up to 8 mole water/mole lipid a lamellae repeat distance constant of about 51 Å, the distance increases linearly to a limiting value of 65 Å between 8 mole and 34 mole of water[162, 169]. The structure up to 65 Å distance is associated with a packing of the lipids in a bilayer[162, 169].

Diffusion measurements[169] with tritium-labelled water shows an increase of the water diffusion coefficient with water content. It reaches a plateau at 11 mole water/mole lipid. Above 21 mole water/mole lipid D increases strongly[162, 169].

The proton magnetic resonance technique (PMR) gives good indications of different movable water structures. Henrickson[170] found with egg phosphatidylcholine

in CCl_4 a big decrease in PMR linewidth $\Delta\nu_{1/2}$ and a decrease in viscosity at 4 mole water/mole lipid (primary hydrate). She concluded that the first few water molecules are tightly bound. With increasing water content the change of $\Delta\nu_{1/2}$ reaches a limit at 12 mole water/mole lipid (secondary hydrate)[162, 170]. – In a similar system: with benzene as solvent, Walter and Hayes[171] observe a plateau of PMR line width $\Delta\nu_{1/2}$ till 2.7 mole water/mole lipid; above this content a linear decrease of $\Delta\nu_{1/2}$ till 12 mole water and at 12 mole water a sharp change of this linear decrease to a smaller slope. The decrease of $\Delta\nu_{1/2}$ was observed with the water signal and with the $N^+(CH_3)_3$ signal also, it indicates an increasing motional freedom of water and the phosphorylcholine group[162]. – The PMR techniques give indications too that Ca^{2+} ions reduce the "bound" water molecules on phospholipids[162] and on a fast exchange between the bound and bulk water[162, 172]. There are three different states of water on phospholipids: bonded, trapped between the bilayers and bulk water. The bound water of the primary hydrate 1–6 water shows with deuteron magnetic resonance a rotational correlation time of $\tau_c = 10^{-7}$ sec, the secondary hydration shell to 11–12 water/mole lipid has: $\tau_C \lesssim 8 \cdot 10^{-10}$ sec and the trapped water to 11–13 water/mole lipid: $\tau_C < 3 \cdot 10^{-10}$sec[162].

The NMR rotational reorientation time of liquid water at 25 °C is 2.5 10^{-12} sec and the dielectrid relaxation time of liquid water is $8 \cdot 10^{-12}$ sec[173].

Hauser[174] differs from the point of view of NMR-methods – three different water types in lipids: 1. First hydrate 1 H_2O; 2. bound shell 11 H_2O; 3. trapped water 11 H_2O. Together there are 23 water molecules different from bulk one[174]. Compare the secondary hydrate with polyethylenoxids (p. 143) about 20 per O-atom. Kaatze[175] has observed in aqueous solutions of polyvinylpyrolidone (PVP) an increase of the dielectric relaxation time τ with increasing concentration till a factor of 5. He co-ordinates this increase with the slower mobility of hydrate water. At 6–10 mole PVP/kg water τ reaches a constant level. Kaatze assumes micelle forming in this c-region[175].

The agreement between the hydration numbers of lipids determined with different methods and with different hydrocarbon chains or with determinations of the liquid crystalline phases or of the micelle solutions in organic solvents is considerable. It shows that the assumption of different types of water starting with a bonded one on lipids is very useful. The amount of bonded water (average value 12.5 mole/mole phosphatidylcholine) agrees with the quantitity of unfreezable water determined from calorimetric methods[162]. NMR measurements with D_2O indicate a small but significant difference of the H_2O or D_2O interactions with lipids. With D_2O a deminution of the second hydration sphere to one water per phosphatidylcholine molecule was observed[162, 171]. The linewidth of $N(CH_3)_3$-group decreases with increasing exchange of H_2O with D_2O too[162, 171]. Gary-Bobo[176] has observed on lecithin with the tritium technique a maximum of the diffusion co-efficient of water at 11 H_2O per lecithin molecule and a second smaller maximum at 16 H_2O per lecithin in the case of fatty acids. He concludes that bulk water can be observed at $>$ 24 H_2O per mole lecithin units[176].

Monoglyceride-water mixtures form during cooling below the concentration, depending transition T_c coagels or gels with lipid bilayers and water between[162, 177]. 1-monolaurin-water shows by the phase diagram and by NMR data a monohydrate[177]. With increasing water content the melting point T_m decreases till a saturation content

of 13 mole water/mole monoglyceride[177]. There is a small T_m maximum of a bihydrate. There are three phases: melt, liquid crystalline and solid. The melting point of the melt decreases with water content x_1, but the transition T: liquid-crystalline/melt increases with x_1. Water "acts as a very good cement between the molecules to which it is hydrogen bonded"[178] in the liquid crystals. The experiments on monoglycerides indicate: 1 mole H_2O is strongly bound; there is evidence of a second hydration layer of 1–3 mole H_2O/mole monoglyceride; the amount of trapped water is about 10 mole; the total amount of saturation is 13 mole water/mole monoglyceride[162].

b) Nucleic Acids, Peptides and Proteins

The properties of protein in aqueous systems depend on[179]:

1. intramolecular interactions (non-bonded, internal rotation);

2. solvent-polypeptide interactions (H-bonds, hydrophobic bond, solvent binding).

The solvent effect upon conformational stability depends on[179]: 1. bulk effect acting as a dielectric medium; 2. specific interactions with polar polypeptide groups (NH, CO);

3. changing of interactions of polypeptide groups by solvent molecules nearby (hydrates, hydrophobic interactions). – A simple model on the water effect on H-bonds of amides is the dimerization of acetamide with a H-bond energy of – 5.1 kcal/mole in CCl_4 and 0 kcal/mole in water[180].

A larger review on water/nucleic acids etc. is given by Eagland[181] with the stress on salt effects. Some problems are similar to those of the lipids. There are some observations on non freezable water related to "bound" water[181, 182]. The influence of water on the conformations of proteins or polypeptides is important too. Eagland[181] classified the conformations in two main groups: in the first, interactions between peptide units predominate over peptide/solvent interactions (native helical configuration), in the second group the interactions peptide solvent predominate (denaturated random coil configuration[181]). The helix-coil transition is T-dependent. In the case of a 36 residue peptide from rat skin gelatine the heat of activation of the transition is 18 kcal/mole[181]. The main stabilisation factor of the native configuration are the amide-H-bonds, but H-bonds with hydrate water may play an important role too. A lot of crystal structures of biochemical or biopolymers with specific H-bonded hydrates[183, 184] are known.

By the NMR-technique[182] the following hydration numbers for different amino acid side chains were found: Glu^- 7.5; Tyr^- 7.5; Asp^- 6; Lys^+ 4.5; Lys 4.5; His^+ 4; Tyr, Arg, Pro 3; Asp, Glu, Ser, Trp 2; Ala, Gly, Val 1. The influence of charges on the hydration numbers is distinct. – The ordering effect on water by gelatine gels could be determined by the enlargement of the dielectric constant[185, 186]. Bull and Breeze[15] have concluded by isopeistic investigations that 1 mol of egg albumin binds 740 mole of water. Added ions dehydrate the protein partially[187].

Privalov and Mrevlishvili[14, 188, 189] made calorimetric studies on collagen. Up to 0.3 g water/g collagen is strongly bonded, this water shows no crystallisation until at liquid He T. No phase transition in collagen containing 2–3 mole water/100 g

collagen was found by NMR technique either[3, 183, 190]. Transitions of collagen from helix into the disordered state of statistical coils is accompanied by "melting" of ordered water chains. Heat capacity studies[14, 188, 189] have demonstrated that helical and coil forms of collagen are characterized by the different mechanism of water binding with different topology of water. NMR data have shown that water near collagen is much less mobile than in bulk water[14]. A similar observation resulted from microphotometric studies in the near IR in my own laboratory[191, 192]. Figure 30

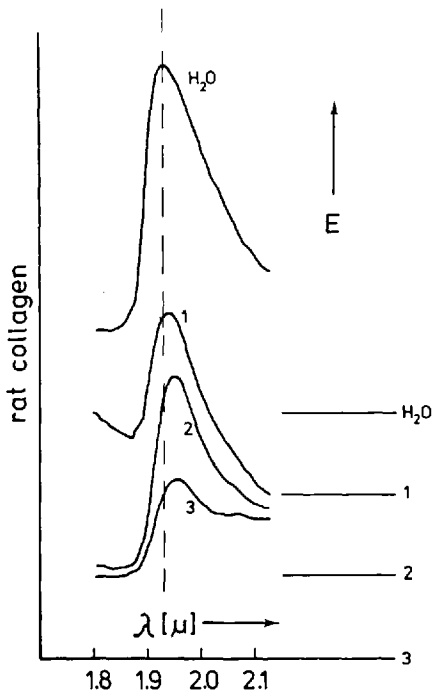

Fig. 30. Upper: Combination band $\nu_1 + \nu_2$ of water. Lower: Absorption band of water in collagen during drying[142]. The most dried sample 3 shows a $\Delta\lambda$ similar to supercooled water at – 20 °C

shows a combination band of water in collagen fibre during drying. The maximum shifts to longer wavelength like the bands of bulk water during cooling (see p.[49, 50]). The maximum of water corresponds to supercooled water about – 20 °C. Sidarova *et al.* have published some spectra of tissues of the frog rana temporaria[193]. They observed a small frequency shift of the combination band 2100 cm^{-1} (bending and libration). There are no precise measurements of the T-dependence of this band. We estimate from the published data at cell T of 40 °C: a structure T_{str} of about 30 °C of blood or muscles or brain tissues (without drying). At cell T of 20 °C the T_{str} of tissues is about 15 °C. This result: the first water shells in collagen correspond to supercooling – that means water with a larger H-bond content – agrees with NMR-data on bacterial cell walls, done by Resing and Neihof[19, 194]. The purified cell walls consisting on peptides and polysaccharides were dried and humidified at

76% rel. humidity. The water content is 33% by weight. The molecular NMR jump times in cells were reduced by a factor of 100 compared with liquid water but do not reach the ice values – by a factor of 10^{-6} smaller – (Fig. 31). The maximum of τ in cell walls corresponds to an extrapolated value of liquid water about T = 235 °K. Striking is the broad distribution of jump times of water in cell walls co-extending from times of liquid water to ice. We can compare water in cell walls with supercooled water with a broad scale of mobilities. The reduction of the apparent T may be induced by the interaction water/mucopolysaccharid groups. Water in charcoal with mean pore radius of 13 Å shows a broader distribution of τ but with a

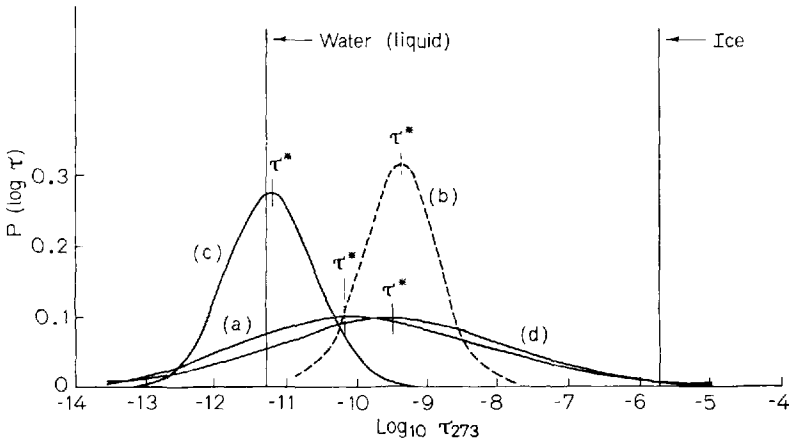

Fig. 31. Distribution functions of the jump time (NMR) at 0 °C for liquid water, ice, adsorbed water on: a) porous glass AG 39, b) zeolite, c) charcoal and d) bacterial cell walls. (Belfort *et al.*[196])

similar maximum like liquid water (Fig. 31). Belfort *et al.*[196] have observed with glass capillaries of 24 Å pore diameter a similar distribution of τ and a value between cell wall water and liquid water (Fig. 31). Glass capillaries with 129 Å and 256 Å showed water mobilities like liquid under saturation conditions[196]. The mobility decreases at 50% rel. humidity to similar values like the 24 Å capillaries[196]. The coal capillaries may have too small an interaction with water. The stronger interaction of the glass walls may reduce the mobility of the orientation defects in liquid water.

The diameter of 24 Å of the glass capillaries corresponds to the diameter of the water aggregates in the simple model of bulk water. From the point of this model one can assume that the strong interactions of the glass wall or the cell walls prevent the flickering process of the orientation defects.

Likhtenstein[197] assumes because of his experiences of spin label method three layers of water-proteins matrix: 1. the deeper layer with relaxation times $\tau_C \approx 10^{-7}$ sec; 2. "the glycerol like layer with $\tau_C = 10^{-8} - 10^{-10}$ sec, built up by side proteins groups and surrounding water molecules in broad 'caves' "; 3. the bulk water located at distances of about 12–14 Å from the surfaces. According to Mößbauer spectroscopy

results in active centres iron containing enzymes, Likhtenstein[197] assumes two modes of the protein local mobility: 1. $\tau_C = 10^{-8}$ sec and amplitudes x more than 0.4 Å; 2. $\tau_C = 10^{-7}$ sec and x = 5–6 Å. This second mobility requires a critical magnitude of rel. moisture (0.4)[197].

Lumry[198] stresses from the view of hydrogen exchange of globular proteins: mobile defects (poor binding) in proteins. "A high mobility of the defects through the protein provide channels for water migration to inner sites to provide binding sites for substrates and to assist in the catalysis process"[198]. "Proteins must be viewed as dynamical rather than static systems"[198]. For H-exchange in lysozym Lumry gives activation enthalpies, about 5 kcal/mole[198, 199]. The same value is given for acetyl glucosamine on lysozyme[198], this energy corresponds to the activation energy for water viscosity or approx. to the H-bond energy of one OH group.

Mrevlishvili[189] gave the following enthalpy differences for the helix-coil transfer of collagen: fibre → coils/solution (5 kcal/mole); coils/solution → helix/solution (−5.5 kcal/mole); helix/solution → helix/fibre (0.5 kcal/mole). The following table gives the differences between the hydration water of native and denaturated protein[181, 188]:

Table 6.

Protein primary hydration	Native conformation g H_2O/g protein	Denaturated conformation g H_2O/g protein
Tropocollagen	0.47	0.51
Serum albumin	0.49	0.49
Egg albumin	0.31	0.33
hemoglobin	0.32	0.34

Buffer ions increase the water content in both conformations[181, 188]. The hydration of 0.465 g/g collagen corresponds to 2.4 H_2O per residue[181]. Kuntz[182] has given by NMR data 2.7 H_2O. In addition to the primary hydration (Table 6) there is an extended region up to 50 water molecules of slight restriction of the motional freedom[181, 188]. The larger region of restricted water molecules determined by calorimetric data are confirmed by dielectric data, which indicate a long-range ordering of water around gelatine[185].

Andronikashvili[200] arrived at the following conclusions using the method of low T calorimetry: 1. During the embryo growth (silkworm eggs) the fraction of "bound" – unfreezing water decreases, reaching the value characteristic of most normal tissues (0.2–0.4 g H_2O/g dry substance); 2. Tumor tissues have twice as much unfreezing-bound water as normal ones (0.57 g instead 0.36 g NMR data establish the calorimetric data; 3. In tissues one can differentiate between two fractions of water: a) "nonrotating-hydrated" with a regular structure, b) external hydrate layer not being stereospecific. Between both fractions exchange takes place; 4. At heat denaturation, a destruction of the internal hydrate layer, takes place. The total hydration in coil state increases (for collagen: native 0.5 g H_2O in statistical coil, 0.9 g H_2O per g collagen); 5. Conformational transitions of DNA from the A into the B-form results

in a narrow range of values of water activity; 6. The bound water performs a functional role in the norm and pathology of cell elements[200].

Calorimetric data on native DNA shows similar hydration properties like on collagen[188]. Up to 0.6 g water/g dry DNA water is unfreezable. The amount of bound water corresponds to 11.6 mole water/molecule nucleotide. Similar amounts are found by NMR technique[181]. Addition of 0.15 mole/1 NaCl reduces the bound water content to 10.5 – With calorimetric studies on denaturated calf thymus DNA was found[181, 201], a dependence of the unfrozen water on the additions of cations: Li 30, Na 24, K 22, Cs 14, Mg 17.

Falk *et al.*[202] have shown by IR technique that NaDNA (B-form) is only stable in the solid form above 75–80% humidity. At 80% humidity all specific hydration with 10 mole water per mole nucleotide is occupied. Above 92% rel. humidity IR bands of bulk water appear. In the range of 80–55% rel. humidity 4–5 mole H_2O/mole nucleotide are successively removed. Below 55% rel. humidity a general loss of helical structure occurs. At low T till 10 water/nucleotide show no freezing (inner hydration layer). A second hydration layer of 3 water shows partial freezing[181, 203]. – The NMR spin-echo technique shows a shortening of the relaxation time of water in calf thymus DNA indicating less mobile hydrates of 10.4% by weight. – The results of Falk *et al.* indicate the role of stable hydrates in the structure of biopolymers[202].

Different techniques indicate in the case of DNA the following hydrate spheres[181]: 1. two 2. ten (first hydration shell) 3. secondary shell till 50 water. The secondary shell is able to solve cations[201]. Compare our experience that the secondary hydrates of polyethylenoxides contain ions (p. 149). There is one NMR experiment showing that the primary water bound to protein did not exchange within a period of 24 hours[204]. Koenig assumes a layer of 10–120 water around the protein which make their Brownian motion together with the protein[205]. Secondly there is weakly bound water which exchanges with bulk water in the order of 0.1–10 μsec[205].

Berlin *et al.*[206] have found with the differential scanning calorimetry an increase of the heat of vaporisation ΔH of water from 10.2 kcal/mole (liquid like) to 12.9 kcal/mole (larger than ice-like), with increasing water content from 0.1–0.45 g water/g protein in casein, collagen, β-lactoglobulin and bovine serum albumin. The increase of ΔH with water content may be induced by some water molecules at low concentrations with only one OH group bonded to proteins or that special structures for example, a helix depends on a stoichiometric water content. Compare the viscosity maximum of PIOP-9 at a certain water content[31]. Berlin, Kliman and Pallansch[206] give one measurement with a smaller water content of 0.055 g water/g bovine serum albumin and found ΔH = 12 kcal/mole. It would be necessary to make more experiments to prove if this indicates stabler primary hydrates. One example with water of different H-bonds to protein is given by a crystal analysis of carboxypeptidase[183, 207]. There are two H_2O with 4 H-bonds, seven with 3 bonds and one with 2 H-bonds[183, 207].

The unfreezable water may have more than a theoretical meaning. Adding more water than the total of the unfreezable to biopolymers, small amounts of freezable water are observed but the freezing point is reduced (see Figs. 16 and 20 in [181]). So in fish (cod) muscle water freezes at $-10\,^{\circ}C$![208]. Feeney has found a special anti-

freeze glycoprotein in antartic fishes[209]. This may be a strong hydrated protein like the technical antifreeze auxilaries that consist mainly of polyethylenoxid products whose strong hydration we could show. Small additions of polyethylenoxids without hydrophobic end groups with large molecular weights (about 1 million) make water highly viscous. It is possible to draw viscous fibres from these solutions with diameters of about 1/200 mm. The possibility of reducing such small fibres against the surface tension may be induced by the strong hydrate forming of the polymers. These solutions reduce the turbulence of moved water. Experiments were made to accelerate moving ships with these additions. A lot of sea animals have viscous polymers on their skin. It may be that nature needs this effect.

c) Ion Effects on the Hydration of Nucleic Acids, Peptides and Proteins

Addition of electrolytes stabilize the helical form of DNA[181] Eagland thinks there are two concentration regions: the first below 0.1 mole/l; the second above 0.1. The distinction of these two regions should depend on the Gouy-Chapman doublelayer. For example, the reduced viscosity of gelatine solutions have a maximum of approx. 0.04 mole NaBr/l[210]. The melting temperature T_m of the helix-coil transition of DNA increases with added ions in the series. $Li^+ < Na^+ < K^+ < Rb^+ < Cs^+$. The ion series depends a little on the concentration. This common effect we could observe with the structure T, determined by the IR method and the equilibrium constants of the micelle formation of polyethylenoxides too. As the spectroscopic method of the water structure have shown (Chapter IIa) the anions in the Hofmeister series have the major effect on T_m of DNA[181, 211] too: $CCl_3COO^- \gg CF_3COO^- > CNS^- > ClO_4^- > Ac^- > Br^- > Cl^- > HCOO^-$. A similar series is found on the solubility of the bases thymine, adenine etc.[212]. $Cl_3COO^- > CNS^- > ClO_4^- > J^- > Br^-$ have salt-in effects, SO_4^{--} Cl^- show salt-out effects[181, 212]. Salt-in effects were observed on acetyltetraglycine ethyl ester too in the series[213]: $SCN^- > ClO_4^- > J^- > NO_3^- > Br^- > Cl^- > NH_3COO^-$. The ions most effective in salting-in peptide groups show the least effective salt-out effect on the alkyl side chains[181, 214] in the series: $NaSCN < CsCl < NaClO_4 < LiCl, KCl, NaCl, NaBr < KF < CaCl_2 < Na_2SO_4$. Salt-out effects on peptides were observed in the series[215]: $Cl_3CCOO^- < SCN^- < ClO_4^- < J^- < Cl^- < SO_4^{--}$ or on poly-L-proline[216, 217]: $ClO_4^- < J^- < SCN^- < Br^- < Cl^- < Ac^- < SO_4^{--}$; $K^+ < Na^+ \ll Li^+, Ca^{++}$.

The order of decreasing capacity for reducing the optical rotation of poly-L-proline is[181, 216]:

$$SCN^- > ClO_4^- > J^- > Br^- > NO_3^- > Cl^-.$$

The effect of salts on the biopolymers is clearly shown by the results of v. Hippel and Wong[218] (Fig. 32). Salts with possibility of giving salt-in effects reduce T_m of ribonuclease. Salt-out effects giving ions like sulfate increase T_m, they reduce the interactions of biopolymere bulk water. Using models, there are opinions that proteins are preferentially hydrated in the presence of SO_4^{--}, less with $Cl^- > Br^- > CNS^- > J^-$[218]. If this is true, weaker interaction with the bulk water should strengthen the stability of fixed hydrates on the polymers.

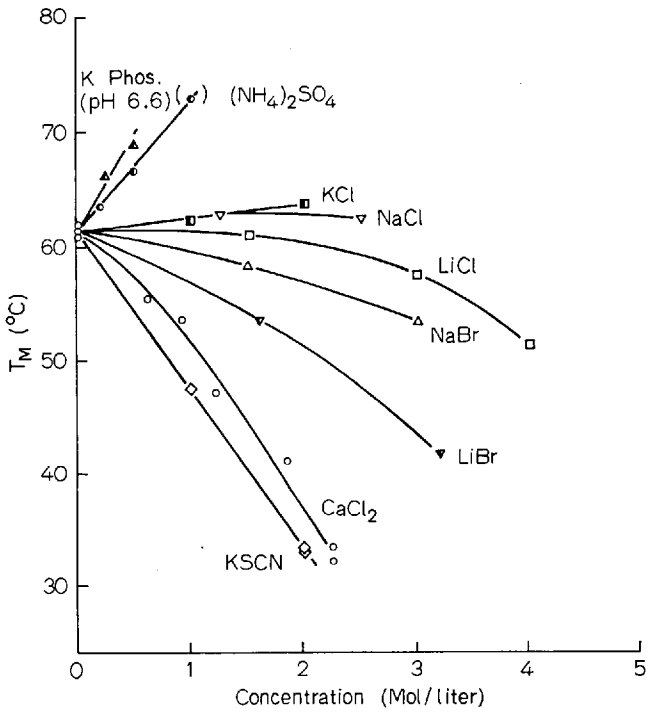

Fig. 32. Transition T of 0.5% ribonuclease as a function of the concentration of added salts. pH = 7 (von Hippel and Wong[222])

Measurements in my laboratory have shown that the force-area diagram of monomolecular films of $C_{18}H_{37}-(O-CH_2-CH_2)_nOH$ with $n = 0, 1, 2, 3$ on water are influenced by salt addition to the water. $NaClO_4$ increases the force to obtain a certain area more as Na_2SO_4[219]. This experiment shows that salt additions influence the properties of organic phases too.

It has been remarked that the ion effects on the stability of proteins are caused by interactions or bindings of the ions to the polymers[181, 215, 220, 221]. But this seems to be very improbable, because the ion series is mainly the Hofmeister ion series. We have shown that the Hofmeister ion series is the series influencing the water structure (see Chapter IIa). In addition the similar ion series obtained with very different polymers or oligomers is a second point in favour of the change of the water structure. This can be substantiated by comparing our very different experiments with polyethylenoxide products.

The common effect of salts can be established by the observation that the influence of different ions on biopolymers is nearly additive[181, 214, 222, 223]. This can be demonstrated by the turbidity point T_T of PIOP-9 (10 g/l) too (Fig. 33)[224]. The uppermost curve (left corner) gives T_T for $NaClO_4$ solutions. The second curve, going down, starting at 64 °C corresponds to Na_2SO_4 solutions. The curves starting at this curve going to the right belong to supplementary additions of $NaClO_4$. The abscissa gives the total amount of salts c. The Na_2SO_4 additions to ClO_4^- solutions

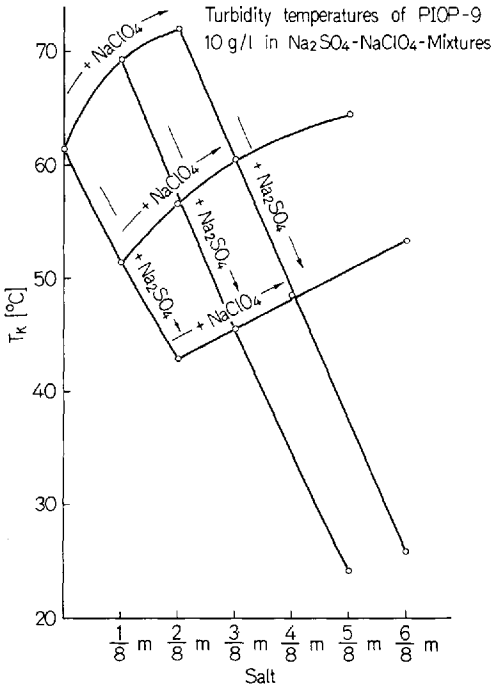

Fig. 33. The additivity of ion-influence of the turbidity point of PIOP-9 (10 g/l) in water[224)]

give in approximation additive effects too. But the nonlinearity of the salt-in effects of the ClO_4^- ion as functions of c decreases with T. The salt in effect of ClO_4^- seems to decrease a little with decreasing T. As an approximation the salt effects in Fig. 33 are additive. Especially salt-out action is additive to salt-in actions.

In Fig. 9 we have shown the importance of the charges of the ions on the water structure. There are some observations of large effects of divalent ions. For example, Andronikashvili[200)] found a considerable change of the physical characterization of water in suspensions of cells in the presence of bivalent ions of metals, for instance Ca^{++} ions. Brandt[225)] has observed a big influence of ions and specially of Ca^{++} on co-operative structural transitions in the erythrocyte membrane[225)]. – Alfsen *et al.*[226)] report on specific changes of the kinetic parameters of the reaction of oxo-steroid isomerase by divalent ions (Ca^{++}, Sr^{++}, Mg^{++}). They think of the change of the water structure as the cause.

A generalisation of the observation of reduced mobility of water or dyes in polyamides by reduction of the water content (p. 152) is the fact that bacteria are living in food with only up to an 85% water content[227)]. A reduction of the humidity from 100% to 85% reduces the rate of bacteria's growth[227)]. NaCl content reduces this rate further. The enzyme activity of yeast is reduced too by decreasing the water content. NaCl or NH_4Cl additions give a further reduction[227)]. Micro-organisms with halotolerance alter the free amino acid concentration and proline content to resist against electrolyte solutions.

d) Polysaccharides

Polysaccharides have a lot of OH groups and in addition other H-bonding groups like –NHCO–, –O–, –COOH COO^- or $-CSO_3^-$. Their hydration plays a dominant role. In addition cyclic structures of mono-saccharides with six membered rings have distances corresponding to the O–O distances in the ice structure. β-D-glucose could exactly replace a chair conformation of the ice water arrangement[228], in D-galactopyranose (component of agar) the $O_1-O_2 \cdot \cdot O_3-O_4$ distances are 2.85 Å[229], similar to the O–O distance in liquid water. The distances of OH and O in saccharides favour hydrates with H-bond angles $\beta = 0$. For example, Fig. 34 shows a model of

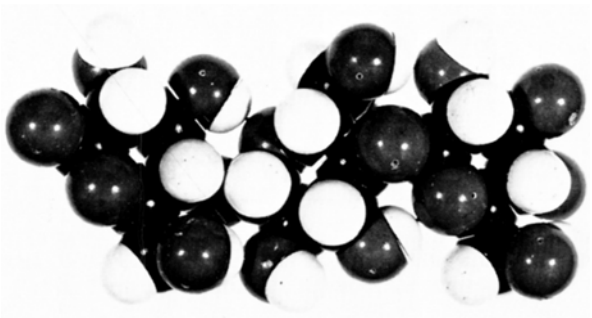

Fig. 34. Model of a cellulose chain with internal H-bonds of angle zero

cellulose with assumed internal H-bonds of $\beta = 0$. As a result the thermodynamic behaviour of glucose solutions indicate that the H-bonds sugar-water are stronger and more extensive than the water-water one's[230]. The apparent molar volumes of mono-saccharides in water are independent of the solute concentration[228, 231]. The sol-gel transition of agar differs only slightly from the concentration[229]. This agrees with the assumption of a strong hydration.

NMR and dielectric relaxation show an increase of the relaxation time of water by a factor 2.5–3 in sugar hydrates[228, 232]. D-glucose should have 6 hydrate water per mole sugar at 5 °C and D-ribose 2–3 mole water/mole sugar[228]. Suggett supposes that the reason for this difference may be the similar distances of the H-bond acceptors in glucose as in ice, and the conformation of D-ribose has fewer similarities with the distances in structured water[228]. Warner[233] has discussed a correlation between the stereochemistry of sugars in relation to their hydration and the biological specificity. For example cyclitol scyllo-inositol has ice-like spacings. Correspondingly, scyllo-inositol is not oxydized by acetobacter suboxydans but myo-inositol is[228, 233]. The latter has less suitable water distances. Otherwise, the loss of order of dehydrating

DNA from E.coil can be prevented by myo-inositol. Warner assumes that inositol may replace the hydrate water and stabilize the DNA structure like hydrate water[228, 233].

The O–O spacings of sugar fit the water-lattice better at room T in liquid state than in ice. Warner gave the working hypothesis that this may help to prevent freezing damage of cells[228, 233].

The water interaction with sugars favours special conformations of the sugars in a different way to organic solvents[228, 234–236]. Polysaccharides form gels easily. This seems to indicate the many possibilities of H-bonding with water. Again the ions influence the gel formation of polysaccharides in the Hofmeister ion series with preferences of the anion[228, 237, 238] and indicate the role of H-bonds in gel formation.

The retardation of the retrogradation rate of amylose is induced by anions in the order of the Hofmeister ion series[228, 239]

$$J^- > NO_3^-, HCO_3^- > Br^- > Cl^- > F^-$$

The retardation corresponds to a salt-in effect by water becoming more hydrophilic by large anions. The same series is obtained by hindering the gel formation[228, 240, 241] because structure-disturbing ions increase the content of non H-bonded OH groups in water.

The rejection of anions by cellulose acetate membranes is a mirror of the Hofmeister ion series again[242]): Citrate > tartrate > SO_4^{--} > Ac^- > Cl^- > Br^- > NO_3^- > J^- > SCN^-. The reason can be understood by our experiments with the influence of ions on the water content in organic systems of two phases: organic/aqueous (Chapter IIe.). The structure breaking ions increase the water content and imply an increase of the bulk water like H_2O molecules which can solve ions (Chapter IIe.).

Ions present during the preparation of cellulose membrans influence the structure of the membrane too in the order of the Hofmeister series[243]. This is to be understood as well by a different water structure in the presence of special ions.

With a model of polyvinylalcohol-derivative membranes Gramain *et al.*[244] have found that the water-flow through membranes depends strongly on concentration and type of added salts with distinct differences between structure-making and breaking-ions. Ions can be selectively transported across membranes using mobile carriers like monensin[245]. Monensin an organic acid can carry selectively Na^+ in presence of K^+ [245]. This mechanism may be related to special affinities with the carrier which would induce a high partition co-efficient.

Polysaccharide gels can be formed with 99.5% water and 0.5 polysaccharide[228]. There are no unequivocal results if the water structure changes much during gel formation[228]. Suggett[228] assumes that "the most reasonable explanation" of NMR relaxation data is a consequence of the slowing down of the rate of exchange of water molecules. This agrees with our conclusion on the pseudo-iso cyanine gels (Chapter IIb.). The mechanism standard keeping the T of the human body constant is unknown. D-glucose monohydrate changes its X-ray diffraction pattern at 37° from the monohydrate pattern to a similar pattern of anhydrous glucose[246].

e) Water in Biologic Tissues

The development of "supramolecular"[247] biology, the biology of large aggregates of biomolecules, has been strongly influenced by two factors during the last years: At first, the so called association-induction hypothesis[c])[248], "which proposes that the cell is a highly structured complex entity and that the cellular transport mechanism is determined by the macromolecular interaction of ions and water"[249]. Secondly, some observations, especially NMR data, were interpreted to the effect that the/or some cellular water is "ordered"[204, 250–252]. For example, the diffusion co-efficient of liquid water at 25 °C of 2.4 10^{-5} cm^2/sec is reduced in mature rat gastorcnemius to 1.4 10^{-5} or in mouse tumor (mammary) to 0.8 10^{-5} [253].

An attempt to confirm the partial ion binding to macromolecules is given by Carpenter *et al.*[254]. They found by a micro-electrode technique that the internal conductivity of plysia neurons is only 5% of the conductivity of squid axoplasm or normal sea water[254]. They concluded that an extensive binding of ions to cellular macromolecules existed.

Ling and Negendank have determined the water uptake of frog muscle from humid air (Fig. 35)[255]. After taking up 3–5% of the total amount, a "co-operative" adsorption of water is observed like a capillar or multilayer-condensation.

We have to look at the mechanism of this co-operativity as having a certain amount of water inside the tissue. Different possibilities are thinkable; dissociation

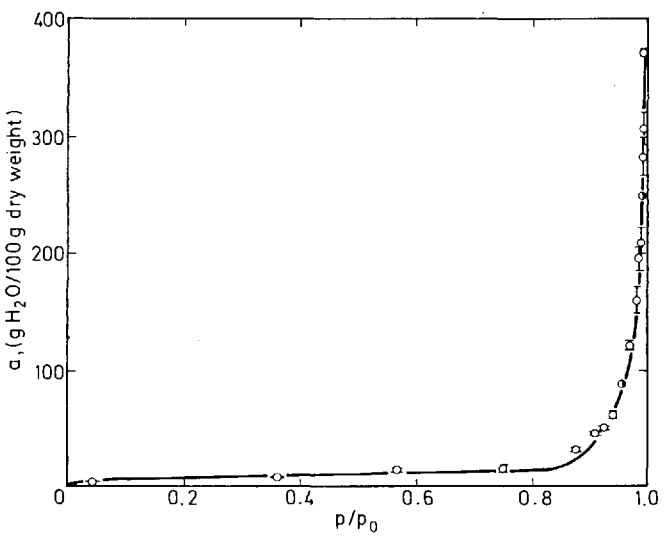

Fig. 35. Uptake of water by frog muscle at various relative water vapour pressure (Ling and Negendank[255])

[c]) Association indicates the three dimensional arrangement of biopolymers which may carry some charge system of ionic groups (dissociation different from a monomeric solution). 2. Induction means an inductive mechanism of charge transfer by association.

of ion groups, finishing clathrate-like orders or loosening the system of the biopolymers H-bonds or destruction of crystallisation. Ling and Negendank see a verification of their two different observed adsorption mechanisms: in the fact that different other techniques observe two water fractions in tissue – neither has the property of normal water –. The larger fraction exchanges its water more rapidly than the smaller fraction. A smaller fraction is given as: 8% of the total water in rat muscles (NMR data by Hazlewood *et al.*[256, 257]; 10% in frog ovarian eggs (diffusion label technique by Ling *et al.*[258]); 3% in rat brain (NMR, Cope[250]); 27% in rat voluntary muscle (NMR Cope[250]). Chirgadze *et al.* have observed in procollagen films a dichroism of the IR overtone band of water (5100 cm^{-1}). In rat tail tendon collagen this dichroism reaches its maximum at a humidity of about 60%, the maximum amount of the protein is in the ordered "crystalline" form at this humidity[259].

Similar water adsorption curves were found by Bull on collagen, gelatine, egg albumen, β-lactoglobulin, serum albumin, silk and nylon[260]. Bull has found that the adsorption isotherm can be plotted theoretically by the BET theory up to 50–60 rel. humidity[248, 260]. At higher concentration the Bradley theory fixes the data[248]. This theory assumes a stronger interaction of the adsorbed particle. The evaluation with the BET theory shows that the monomolecular surface film corresponds to a hydrate of 1 water per protein residue (obtained at about 0.1 rel. humidity) and the condensed film corresponds to a dehydrate (obtained about 50–60% rel. humidity). A specific collagen hydrate structure was found by X-ray diffraction[183, 261]. In this structure two water molecules per three amino acid residues are binding two different protein chains of the collagen threefold helix. Espisoda and Chirgadze have

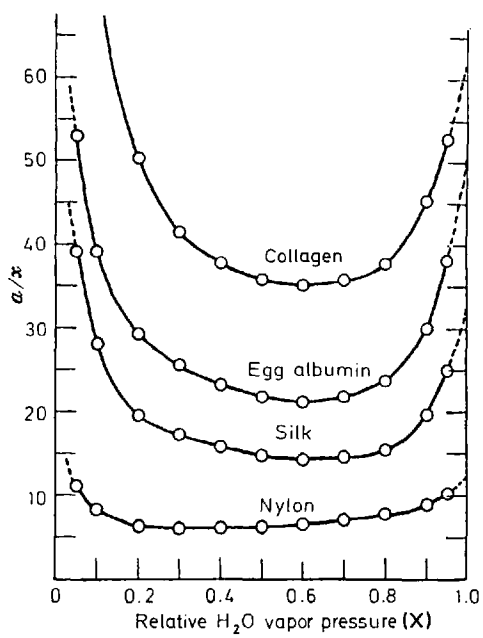

Fig. 36. Plot of water uptake a over relative aqueous vapour pressure x against x for unstretched nylon, silk, unlyophilized egg albumin and for collagen at 25 °C (Bull[260])

shown by X-ray studies that water is "a specific structural element of the collagen helix" too [262]. The absence of water in collagen leads "either to disordering or to complete destruction of the specific configuration"[262]. The different affinity of water to proteins as function of the rel. humidity is shown in Fig. 36. The Figure gives the partition coefficient a/x (a: gram water/g protein; x rel. H_2O-vapour pressure). Until the validity of the BET theory at 0.6 rel. humidity the curves of Fig. 36 correspond to a theory of partition of acids between water and 6-polyamide[15, 263]. At low concentration a reaction between the acid and the small amount of polyamide amino-end groups occur. After saturation a normal partition occurs[15, 263]. The updown slope of the water partition co-efficient in proteins indicates a third mechanism.

Similar adsorption isotherms of water were observed by Falk *et al.*[202, 264] on Na-DNA of calf-thymus. Parallel to the change of the isotherm above 60% rel. humidity Falk *et al.* found an increase of IR dichroism in a humidity region of 60–80%. In addition they have observed a decrease of the UV absorbance at 2600 Å in this region[202]. X-ray results on Na-DNA have shown that DNA above 85% rel. h. is a helical duplex in which the planar purine-pyrimidine base-pairs are stacked perpendicularly to the axis of the B-helix. Between 80–75% r. h. a transition to a helix form occurs with tilting of the base pairs. By lowering the r. h. from 75% to 55% a "disordered state is obtained". The experiments of Falk *et al.*[202] agree with the X-ray results[264]. Above 80% rel. h. Na-DNA is in an ordered phase and below 60% is a disordered state. Water seems to favour the helix configuration of DNA. The appearance of the helix changes the adsorption mechanism from a simple BET to a Bradley one. Falk *et al.*[202] co-ordinate the deviation from the BET theory above 80% r. h. to an expansion of the DNA. After filling the space available with the incoming water further hydration pushes the DNA molecules apart. X-ray diffraction spacings begin to increase sharply between 80–85% r. h.[183, 202, 263]. The adsorption isotherm of collagen also fits the theory of Guggenheim, who corrected the BET theory in the case that the interaction energy between the adsorption layers differs[183, 265]. A "jump" of the adsorption enthalpies of collagen, bovin albumin and casein from − 10 kcal/mole below 1 mol H_2O per 100 g protein to − 12 kcal/mole above 1.2 mol H_2O per 100 g is given by Berlin *et al.*[206]. The water content per nucleotide in Na-DNA has been: 1 at r. h. = 0.1; 5 at r. h. = 0.6; 10 at r. h. = 0.8 about 20 at r. h. = 0.9[202]. Falk *et al.*[202] suppose that between 100–80% r. h. "all the hydration sites of DNA molecule are still filled, and the conditions for the stability of an ordered helix are still fulfilled"[202].

Falk *et al.* have observed a frequency shift of 1240 cm^{-1} PO_2^- group of Na-DNA with increasing humidity[202]. Above, at 65% r. h. a single plateau is reached. They interpret this observation by the fact that above 65% r. h. the complete hydration of the PO_2^- groups is reached. Phosphate groups remain largely hydrated between 75–55% r. h.[202].

The adsorption isotherms give a key to the understanding why grain in air at r. h. about 65% show no sign of life. Otherwise Falk's paper shows the ability of nature to change the structure of DNA which is necessary during multiplication processes. Is the amount of water the key to the growing of the organism at command?

Ordered water seems to substitue the suggestion of a special vital power. "That part of the bound water in living muscles was . . . irreversibly released in death"[260, 267].

The concept of ordered water rests on the fact that a lot of experiments can be consistently classified on the basis of these assumptions. But we should not forget, that the interpretation of some experiments on heterogeneous systems can work only with assumptions. Therefore, we cannot make absolute statements today, we cannot exclude that wishful thinking has influenced some interpretations.

For example, there are some difficulties for NMR data; for example: the macromolecular structure inside the cells compartmentalize the cellular water and violate the validity of the theory of the spin-echo technique[249] or paramagnetic impurities disturb NMR signals. Other difficulties of NMR technique in biopolymers are: distribution of correlation times, exchange with specific binding sites, proton exchange processes, spin coupling with macromolecular protons, heterogenety on macromolecular scale[249].

There are also some indications by NMR data that Na^+ in cells exist in two different states: 60–70% is complexed with macromolecules – and can give special effects by high affinities (see p. 165) – and a rest is solved in structured tissue water[183, 206, 247, 268]. But Behrendsen and Edzes[269] think that this in an artefact by the field gradient fluctuations of macromolecules that contain charged groups. – We have to stress too – relating to the result of the IR microscope technique on collagen – that H-bond band observations can be disturbed by Fermiresonance band overlapping etc. Now, we are busy to look if intensity changes of the symmetric and the asymmetric OH vibration can induce artefacts. – In agreement with our collagen observation Falk *et al.*[202] have reported qualitatively that the OH fundamental frequency of water in DNA is found to increase with increasing r. h. They also concluded "that the average strength of H-bonding decreases as subsequent water molecules absorb"[202]. –

Today, there is a tendency to believe that experimental artefacts can be neglected. There are some proofs which indicate that the two parameters, including the ordered water are correct models. This was the result of a congress of the New York Academy of Science 1972[270]. For instance, Vick *et al.* conclude as a result of extensive studies that: magnetic inhomogeneities of the spectrometer, nonuniform packing of the muscle sample in the probe sample of the presence of large polymers are of minor importance to the NMR results on biologic sample[271].

For example, Swift and Barr established the proton NMR data with ^{17}O relaxation studies on frog skeletal muscles[272]. They also found that the water relaxation is enhanced in muscles in comparison with pure water[272]. Cooke and Wien[273] observed on partially dried rabbit psoas fibers two phases of muscle water: "a small phase, less than 4–5% of the total water, which interacts strongly with the proteins and has short relaxation times; and a major phase . . . with longer relaxation times" a major fraction of the intracellular water exists in a less mobile form than water in a salt solution"[273].

Cooke and Wien have found that the NMR proton chemical shift in muscle water is similar to that in an aqueous salt solution[273]. The relaxation times are different. But the activation energy of the diffusion co-efficient of cellular water in muscle

water is 4.9 kcal/mole, it is also similar in pure liquid water. This clearly shows that ordered water cannot mean water with H-bonds. The interaction energies differ much from the H-bond energy in bulk water. It may mean more a change of the H-bond content or a different structure: water: hydrate or clathrate-like orders.

Privalov has established that the denaturation of proteins (DNA, egg, albumin, chymotrypsin) is endothermic[274]. He recognizes this as an indication that breaking of H-bonds should dominate the denaturation processes[274].

Garlid reports[275] that 30% of matrix water in rat liver mitochondria is bound. He estimated a thickness of about 25 Å of the bond water layer. This value is similar to the extension of the H-bonds in bulk water and would mean in a simple model that the biopolymer surface prevents the flickering of the defects in water. Garlid has found too that the bound water on mitochondria is a solvent of H-bonding organic molecules[275]. If his interpretation is correct, solutes in the secondary hydrate sphere would get more immobile. The time for transfer in cells is increased. –

Some virus proteins crystallise in aqueous dispersions[276, 277]. Some authors have supposed that this effect seems to be typical for biopolymers. But we could show that this is a common phenomenon. Aqueous dispersions of small plastic spheres (about some 1000 Å diameters) crystallise too if their particle sizes are homogenious[277, 278]. The crystallisation could be observed by polystyrene and by polyacrylester polymers. The crystal type could by determined by Bragg reflexes with visible light as a cubic densed package[277, 278]. The particles have charged groups at the surface which give a repulsion term in the potential energy. The summarized van der Waals attraction of large particles (Hamaker) give the attracting forces[279]. Added ions change the lattice constants of these polymer crystals[280].

For example the 111 reflex of the normal latex has a distance of 4370 Å, with additions of 0.8 mole Na_2SO_4 (neutralisation effect on the repulsion term) the distance was reduced to 4390 Å. Similar properties seem to have some virus proteins with their crystallisation phenomena. (Necrose virus and Rothamsted virus).

Summarizing Chapter 3 we can conclude: there are many indications that biopolymers may have different hydrations: 1. Primary hydrate 1–2 H_2O per unit; 2. bonded water ≈ 10–20 H_2O; 3. secondary hydrate shells up to ≈ 50 H_2O and 4. bulk water. The secondary hydrates may solve ions.

The ion effect on aqueous systems of biopolymers can be ordered by the Hofmeister ion series which could be recognized spectroscopically as a series of the change of the water structure.

IV. The Role of D_2O

1. Biologic Observations

In the last chapter we will give a short review on the strong effects on biologic systems by replacing H_2O by D_2O (see review[7]). Bacteria and fungus show a retardation of growth with D_2O amounts between 40–90%[281–283]. An adaption mechanism on deuterium were observed with *E.coli*[284], the fungus Saccharomyces cerevisiae[283]

and chlorella algea[285]. After accustoming to D_2O, algae have difficulties again by returning to a H_2O medium[286]. Protozoa live in D_2O concentration up to 15–20% but die at high concentrations[287]; they can adapt D_2O slowly[288]. The effect of D_2O depends on the age of the micro-organism. At low D_2O concentrations the appearance of very big cells could be observed[288].

Micro-organism have a T region with favoured growth. This region is different in H_2O and D_2O[7, 289, 290] (observations on algae and virus[291]). Mainly with D_2O life at higher T seems to be favoured[7]. This may be correlated with a higher strength of H-bonds in D_2O.

An accustoming of higher plants is not possible in D_2O concentrations $> 70\%$ [7]. There is a retardation effect on the plant's seed growth[7]. The growth of plants becomes retarded with D_2O, irreversible damage occurs after 8 hours in concentrated D_2O, treatment. Peppermint plants show histologic changes of cells and leaves, if they grow in 70% D_2O. The resistance of plants to D_2O treatment increases after some generations[292]. – The lifetime of mice is reduced by D_2O [293]. The damage starts with passivity. Animal organs are harmed, especially organs with a high cell division rate[7]. In animals and plants the kinetics of enzyme mechanism are disturbed by D_2O. The experiments at lower D_2O concentrations have to take in account the equilibrium between H_2O, D_2O and HOD.

2. Biochemical Observations

The intermolecular forces of D_2O are a little higher than in H_2O. Compare the melting point of +3.8 °C and the heat of melting H_2O: 1.43 kcal/mole, D_2O: 1.51 kcal/mole. From this view it is easy to understand that the T-dependence curve of the optical rotation of D-glucose is transferred to higher T in D_2O. The T of the maximum rotation shifts at 5 °C[246] (comp.[294]). The solubility of polar molecules like amino acids are lower in D_2O[295]. The critical micelle concentration of surfactants is decreased too[295]. The denaturation T of ribonuclease increases in D_2O at 4.2 °C [296, 297]. [The gel formation of pseudo-iso cyanine-chlorid starts in D_2O at a 3–4° higher T than in H_2O (Fig. 16, inflexion point $\Delta T = 3.8$ °C)]. The diffusion velocity of D_2O is lower[7]. During the exchange of H_2O by D_2O cells get a water leak and reverse a water excess[7, 298, 299]. Nerve cells show a reduction in the pulse velocity. A reduction could be shown in vitro of the reaction velocity by enzymes[7, 300, 301]. The dissociation constant of D_2O is different too[7]. Therefore, the pH dependence of some equilibria is different in D_2O[7, 302]. Lewin[13] recognizes the higher surface tension as one cause of the D_2O effects in biology. But remembering that the molar surface tension is a measure of intermolecular forces also, this seems to be only a different nomenclature. Another difference between H_2O and D_2O is the lower intensity of the IR overtone bands of D_2O.

Some authors[303] think that below about 27 °C a change occurs in biological activities. For example at 27 °C a cold narcosis sets in etc.[203]. Therefore, an apparent cooling of about 4 °C by exchange D_2O/H_2O seems to be a strong attack on complicated organism.

Generally it can be said, systems with low molecular weight or model systems of polymers show different properties in D_2O too. Therefore, we do not need special mechanisms to understand the influence of D_2O on organism.

> "It is my belief that many of the reactions characteristic of living processes have more to do with the water relationships of the organism than with any other single factor."
>
> R. A. Gortner[204)]

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Note Added in Proof

Related to the hypothetical mechanism of semipermeable membranes discussed on page 40 Siemann in my laboratory has determined water spectra in celluloseacetate – and polyimide membranes. (U. Siemann, Diplomarbeit Universität Marburg, 1976). These membranes are useful for desalination processes. The spectra show a strong intensity of OH vibrations in the frequency region of weak bonded OH groups.

The origin of this absorption may be: water molecules with one OH group bonded to the membrane and the second OH group non-H-bonded or water molecules with H-bonds to weak acceptors of the membranes. In both cases the water transport through these membranes may be related to weak H-bonds water-membrane. In addition the spectroscopic observations show: in celluloseacetate – or polyimide – membranes are less water molecules of the type of liquid water. In agreement with this observation we have found in model glass membranes with low salt rejection (about 70%) at high relative humidity water spectra not far from the spectra of liquid water. As result of these first experiments we may discuss two possible mechanism of membranes for desalination processes:

1. mechanism of page 40: strong bonded primary hydratewater to hydrophilic membrane groups and certain amount of less bonded movable water in secondary hydrates but only few molecules of the type of liquid like water.

2. Movable water molecules weak bonded to the hydrophilic membrane groups and less water molecules liquid-like.

In relation to the discussion of the two types of water in biologic systems: "bonded and unbonded" the spectra of water in celluloseacetate membranes at low relative humidity (about 10%) show some indications that the first water molecules penetrating in these membranes have a higher portion of stronger H-bonded structures in comparison to the properties at higher rel. humidity.

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Medicinal Chemistry

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Contents

I. Introduction

All living organisms contain one or more aqueous phases separated from the environment and from other solutions by membranes. It is in these aqueous phases that the reactions essential to life are carried on and continued existence of an organism depends, among other things, on constantly maintaining the compositions of these phases within sharply defined limits. In these processes inorganic cations and anions play vital roles. Thus sodium, potassium, magnesium and calcium ions are needed individually to maintain the osmolarity of biological fluids, the structure and function of cell walls, the synthesis of protein, the conduction of nerve impulses and the contraction of muscle. In addition, calcium phosphate makes up most of the skeleton and teeth of animals, and calcium carbonate is a major component of the shells of molluscs. Trace amounts of a number of metal ions, including iron, copper, zinc, cobalt, manganese and molybdenum, are essential to the activity of many enzymes, both as activators in enzyme-metal-substrate complexes and as built-in, nondialysable constituents. They are also present as metal complexes in other important biological molecules including haemoglobin (containing iron), chlorophyll (magnesium), the cytochromes (iron) and vitamin B_{12} (cobalt). Recent work with highly pure materials has shown that further metals are essential, mainly at the level of parts per thousand million[1]. These comprise selenium, tin, silicon, nickel, vanadium and chromium.

The range of essential anions is much more limited but it includes chloride, phosphate, carbonate and bicarbonate ions. The first of these is the major anionic species in almost all biological solutions. The other three help to control the pH of body fluids while phosphate is concerned in energytransfer reactions. Trace anions such as iodide and fluoride are also essential.

Although these cations and anions are indispensable, excessive amounts of them are toxic, so that it is important that their concentrations are regulated, either by mechanisms existing in the animal or by externally imposed controls. There are also several kinds of metal ions found in Nature which do not appear to serve any useful biological function but which are highly toxic if they are absorbed into the body. These include arsenic and the environmental pollutants lead, cadmium and mercury ions. Most of the remaining metals occur as inert species such as the aluminosilicates and titanium dioxide that are poorly absorbed, if at all, by plants and animals, or are present in only trace amounts and have little physiological effect.

It is not surprising that early man sought remedies among the things that surrounded him, including inorganic materials, so that the empirical use of inorganic substances in medicine has its origins in antiquity. For example, over 3,000 years ago a decoction of iron (rust) in wine was claimed to have cured the sexual impotence of Iphyclus of Thessaly. In the 4th century B. C., Hippocrates recommended the medicinal use of metallic salts. By the time of Paracelsus many inorganic remedies were known. Nevertheless, it is only in recent times, following developments in chemistry, biochemistry and related disciplines, that logical bases have been established for understanding the roles of inorganic species in medicine. In conjunction with the fruitful concept of selective toxicity[2] this holds promise for the logical

design of inorganic therapeutic agents that are relatively innocuous to the host, while being toxic to unwanted types of cells and organisms, either directly or by depriving them of essential metal ions or other cell components.

In spite of the large body of literature dealing with the mineral metabolism of living organisms, only limited attempts appear to have hitherto been made to bring together what might be described as *inorganic medicinal chemistry*. This topic includes the impact of pharmaceuticals on the mineral composition of cells and tissues, the use of metal-containing agents in therapeutics, and the external control of the concentrations of essential and toxic metal ions in living organisms. A major contribution is made by complexing species, particularly chelating agents which, by definition, contain two or more electron donor groups which are able to co-ordinate to a metal ion to form one or more stable (usually 5- or 6-membered) ring structures. There is strong evidence that chelation is the basis of the activities of many natural and synthetic therapeutic agents, including numerous thiosemicarbazones that have been synthesised as potential antimicrobial, antiviral, antifungal and anticancer agents.

Chelating agents may act in various ways. They may seek out toxic metal ions to bind and excrete them as complexes; they may deliver essential trace metals to tissues that require them; or they may serve as selectively toxic agents. Viruses and bacteria, but not their hosts, may sometimes be inactivated by depriving them of metal ions they need for their metabolism or by providing them with toxic metal ions. For antibacterial or antifungal activity it is often advantageous if the metal chelate is lipid-soluble so that penetration of the cell is enhanced. In some cases the ligands (metal-complexing species) may form ternary complexes with metal ions and apoenzymes so that the enzymic reaction is prevented.

Like many previous writers, I have been impressed with the wide distribution of chelating agents among pharmaceutically useful substances but I have refrained from suggesting this property as a likely factor in their activity, unless there was good supporting evidence for doing so. The *in vivo* chelation of metal ions is a strongly competitive phenomenon, as discussed elsewhere in this Review, and many quantitative factors have to be taken into consideration in assessing the metal-binding ability of a ligand in a biological situation.

II. Topical Applications

A number of essentially inert inorganic compounds are used as dusting powders because of their physical properties, to maintain the quality of the skin, especially by diminishing friction and by protecting epithelial surfaces as aid to tissue repair. These include chalk, talc, boric acid and zinc stearate, applied externally, and insoluble bismuth salts, taken internally in treating ulceration of the bowel. The former use of mercural salts or mixtures of mercury and chalk in some dusting powders (and also in some teething powders) sometimes led to acrodynia (pink disease) in infants. More soluble inorganic species, comprising particularly salts and oxides of heavy metal ions,

are applied as astringents and for the mild stimulation of repair to wounds, ulcers and abscesses. Examples of active ingredients of solutions, ointments and lotions include silver nitrate, alum, soluble zinc salts, zinc oxide, basic zinc carbonate (calamine), bismuth subcarbonate and bismuth subnitrate. Most of these act by precipitating protein. This is probably also the reason for the use of alum, silver nitrate and iron salts to arrest bleeding from surfaces, and for the antiperspirant activity of aluminium salts. Aluminium acetate finds application in the treatment of eczema.

Applications of dressings soaked in aqueous silver nitrate solution have significant prophylactic action against infection in the treatment of major burns[3]. A salt other than the nitrate would seem to be preferable in order to avoid possible reduction to nitrite, with resulting toxicity. One such alternative is silver sulphadiazine. Because of its antibacterial action, silver nitrate is also sometimes used in eye drops.

Potassium, sodium and calcium sulphides are applied externally to soften the skin by dissolving the epidermis in diseases such as psoriasis, acne and seborrhea. Mixtures of calcium or potassium polysulphides and thiosulphate behave similarly and are also depilatories. Another common hair remover is barium sulphide, mixed with starch or zinc oxide, whereas selenium suphide and cadmium sulphide are applied topically, in shampoos, to control dandruff.

Purely inorganic species find limited application as antiseptics and disinfectants, one of the commonest of the mild antiseptics being iodine, usually as its solution in aqueous sodium iodide or alcohol. Sometimes, iodine is used as a dispersion in a solution containing a surfactant with detergent properties, such as polyvinylpyrrolidone. These iodophores form weak complexes with the iodine, solubilizing it, but most of the iodine remains available and retains its bacteriocidal activity. Chlorine and the chlorine-releasing substances including sodium hypochlorite solution, chloramine, halazone [*p*-(*N*,*N*-dichlorosulphamyl) benzoic acid] and calcium oxychloride have disinfectant properties because of their toxicity to Gram-positive and Gram-negative bacteria. Other inorganic substances sometimes used as antiseptic agents, but now mainly of historical interest, include the oxidising agents hydrogen peroxide, zinc peroxide, potassium permanganate, zinc permanganate and potassium chlorate. Boric acid is bacteriostatic and very weakly germicidal. Organomercurials find occasional use in cosmetics but they may give rise to contact dermatitis.

III. Uptake of Inorganic Species

1. Neutralization of Acids

Silica and alumina gels, used in the treatment of gastric and duodenal ulcers and hyperchlorhydria, are nonabsorbable antacids. They control the acidity of the stomach contents by buffering the hydrogen ions secreted by the gastric mucosa, so that more bicarbonate ion is generated in the gastric cells, entering the general circulation and leading to metabolic alkalosis. Other antacids having similar actions include magnesium hydroxide, magnesium trisilicate, magnesium hydroxy aluminate (magaldrate), attapulgite, basic aluminium carbonate, aluminium phosphate and

calcium carbonate. In all cases the therapy depends on the neutralization of acid and deactivation of pepsin to alleviate ulcer pain and accelerate healing of the ulcer.

Antacid preparations based on aluminium hydroxide sometimes contain magnesium salts (and carbonate or oxide) to offset the constipating effect of the alumina. This laxative effect of salts such as magnesium sulphate (or citrate) and other saline cathartics such as potassium sodium tartrate is due to their incomplete absorption from the digestive tract so that, by osmotic forces, they retain water in the intestinal lumen.

However, even simple antacid therapy can have unlooked-for effects. Thus, because of the formation of insoluble phosphates, prolonged use of antacids (excepting aluminium or calcium phosphate) can lead to hypophosphataemia and a compensating increase in calcium absorption or mobilisation from bone[4].

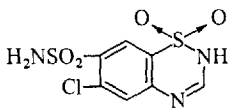
2. Sodium Ion

At its simplest level, adjustment of body composition is achieved by direct intake. For example, sodium ion is the principal cation of the extracellular fluid of the mammalian body, comprising, as the chloride and bicarbonate, more than 90% of the total solute in that fluid. Ingestion of sodium chloride solutions is used to replace salt lost by excessive perspiration. More sophisticated preparations have been proposed for this purpose: one such preparation[5] comprises mainly sodium chloride, supplemented with smaller amounts of potassium and phosphate ions to approximate the average composition of sweat in a sweetened glucose solution.

Longer lasting control of mineral metabolism is achieved by steroid hormones elaborated by the adrenal cortex and by synthetic analogues. Besides their actions on gluconeogenesis, glycogen deposition, protein metabolism and sexual characteristics, the corticosteriods influence *calcium metabolism* and the control of water and electrolyte equilibria, so that profound changes accompany their administration.

Thus, in patients with Addison's disease or other forms of adrenal insufficiency, continuing oral administration of cortisone acetate or fludrocortisone acetate enables salt balance to be restored. Other corticosteriods and analogues that have been used in the hormonal control of sodium levels include aldosterone and deoxycortone acetate. Individual corticosteroids vary in the extent to which they possess the various hormonal activities so that combination therapy is usually required if, for example, mineral balances are to be maintained when corticosteroids are administered for their anti-inflammatory, antirheumatic or anti-allergic properties.

Excessive retention of sodium ions in the body leads to increased release of antidiuretic hormone and a resulting increase in body water. Many diuretics such as chlorothiazide (*1*) and clopamide (*2*) inhibit sodium and chloride ion resorption in the kidney tubules and promote potassium depletion, whereas amiloride (*3*) and triamterene (*4*) diminish the excretion of potassium while causing a loss of sodium ions[6]. Other diuretics such as acetazolamide (*5*) and dichlorphenamide (*6*) inhibit the ion-exchange reaction catalysed by the zinc-containing enzyme carbonic anhydrase.



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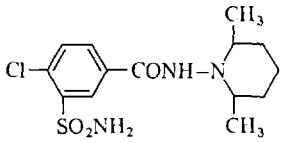

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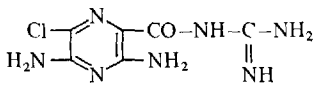

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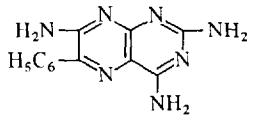

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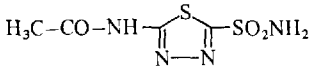

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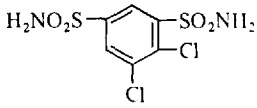

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The pH-buffering of extracellular fluid depends in part on the carbon dioxide/bicarbonate equilibrium so that the intake of sodium bicarbonate is followed by a brief alkalosis and an increased excretion of sodium carbonate in the urine. Depending on its carbonate concentration, the pH of the urine may rise to 8.0[7]. Large doses (80–100 g/day) of sodium bicarbonate were needed if the pH of stomach contents was to be maintained at 4 or over in patients with duodenal ulcers[8]. Oxidation of organic anions in the body to carbon dioxide and water permits the use of sodium citrate, lactate or tartrate instead of sodium bicarbonate. In an analogous manner the ingestion of ammonium chloride induces a brief acidosis as a result of the metabolic conversion of ammonia to urea and lowers the pH of the urine.

3. Potassium Ion

The physiology of potassium excretion has recently been reviewed[9]. Where blood potassium levels are so high as to lead to an undesirable action on the heart, a palliative effect may be possible by the intravenous injection of calcium gluconate. Sodium polystyrene sulphonate, an ion-exchange resin, has been given by mouth or as an enema to rid the body fluids of excess potassium. On the other hand, depletion of the body's potassium reserves can lead to many functional and structural abnormalities including impaired neuromuscular function, changes in gastric secretion and disturbances in electrocardiograms. This may be brought about by the use of diuretics such as chlorothiazide or by an induced metabolic alkalosis. In both cases there is an increased urinary excretion of potassium, leading to potassium deficiency unless the diet is adequately supplemented. Potassium chloride is readily absorbed when taken by mouth as a dilute solution. Care in dosage is necessary because potassium in excess is toxic and many lead to cardiac failure.

4. Lithium Ion

Lithium carbonate (or citrate) is an effective sedative in the management of mania, a severe psychiatric mind disorder[10]. It is used increasingly in the management of manic-depressive illnesses[11, 12], but its mode of action is still unexplained. Possibly, lithium ion reduces receptor sensitivity in extrapyramidal disorder[13]. In many ways lithium ion functions as an incomplete ion substitute for sodium and potassium ions. This affects equilibria and membrane potentials in systems concerned with cation transport and modifies the physiological micro-environment in which cellular reactions are carried out[14].

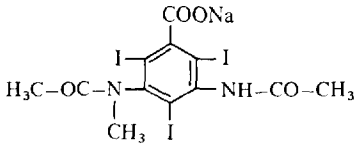
5. Calcium Ion

The major location of calcium in the body is in the skeleton, which contains more than 90% of the body calcium as phosphate and carbonate. Bone resorption and formation keeps this calcium in dynamic equilibrium with ionized and complexed calcium in blood, cellular fluids and membranes. Homeostasis is mainly regulated by the parathyroid hormone and vitamin D which lead to increased blood calcium levels, and by a thyroid hormone, calcitonin, which controls the plasma calcium concentration[15]. Increasing the concentration of calcitonin decreases the blood calcium level, hence injections of calcitonin are used to treat severe hyperalcaemia arising from hyperparathyroidism, vitamin D intoxication or the injection of too high a level of parathyroid extract. High levels of calcitonin also decrease resorption of calcium from bone. Hypocalcaemia stimulates parathyroid activity, leading to increased release of calcium from bone, reduction in urinary excretion of calcium and increased absorption of calcium from the intestine. Urinary excretion of phosphate is enhanced.

When endocrine functions are normal, hypocalcaemia and low-calcium tetany can be treated by giving calcium chloride solution, either by mouth or intravenously. Calcium therapy may also be of benefit in treating osteoporosis resulting from a long-continued negative calcium balance. Care is needed because excessive blood calcium levels can lead to cardiac arrest. A more prolonged intake is achieved if calcium carbonate powder is administered orally. Other materials that may be given to treat hypocalcaemia include calcium gluconate (orally, intravenously and intramuscularly), calcium lactate (orally) and calcium laevulinate (orally and parenterally). Adequate levels of vitamin D in the body are required for optimum transport of calcium from the intestine and for improved differential permeability of the intestinal membranes to calcium[16]. Vitamin D affects the formation of specific proteins that appear to be concerned with calcium metabolism, including a calcium-binding protein in the intestine[17]. It also enhances the movement of calcium out of bone. Conversely, the presence in the bowel of materials such as phytate and oxalate that form insoluble calcium salts decreases the absorption of calcium ion.

6. Contrast Media

Although barium ion is highly toxic, the insolubility and inertness of barium sulphate permits its wide use, given by mouth, as an opaque contrast medium to in-



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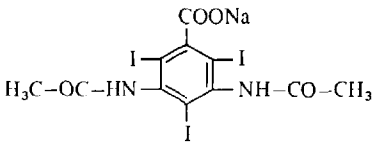

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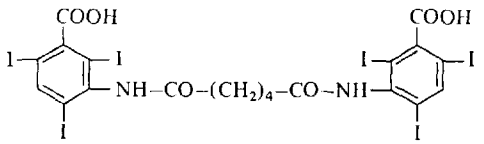

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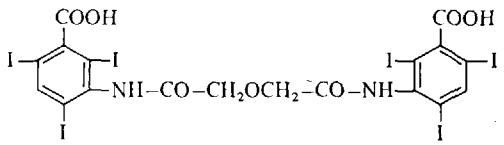

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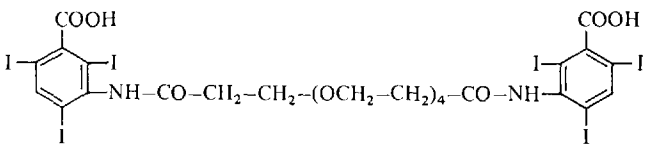

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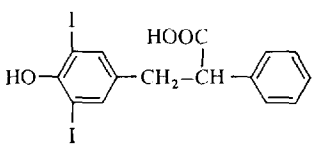

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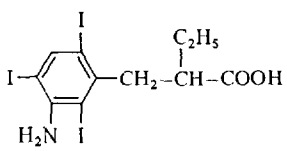

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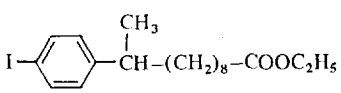

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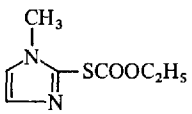
crease the absorption of X-rays as they pass through the gastrointestinal tract. Colloidal solutions of thorium dioxide (thorotrast), formerly used for this purpose, are unsuitable because slow and incomplete elimination from the body may result in the development of malignant diseases. Organic iodides are also used as radio-

opaque agents to delineate other body cavities. Those designed for application to the urinary tract or the vascular system are salts of moderately strong acids, dissolving in water to give solutions of comparatively low viscosity and readily excreted by the kidneys. Examples include sodium metrizoate (*7*) (given intravenously for angiocardiography) and sodium diatrizoate (*8*). Numerous media are available for visualising the gall bladder and the biliary tree. Iodipamide (*9*), its bis (*N*-methylglucamine) salt (cholografin meglumine), and ioglycamic acid (*10*) are currently popular but new compounds such as iodoxamic acid[18] (*11*) iodoalphionic (*12*) and iopanoic acids[19] (*13*) are claimed to be superior. Special applications include the use of ethyl *p*-iodophenylundecylate (*14*) for myelography, and a number of other fluids for examining the bronchial, gastrointestinal and reproductive tracts.

7. Halide Ions

Sodium and potassium *iodides* find limited use as expectorants but a much more important use is as additives, at levels around 5–100 μg/g to table salt in many countries as a prophylactic against goitre. This is a condition arising from iodine deficiency with the result that there is insufficient synthesis of the iodine-containing amino acids, thyroxine and 3,3′,5-triiodothyronine, that are essential components of the



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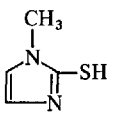

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thyroid protein thyroglobulin. Conversely, potassium perchlorate and thiocarbamide derivatives such as carbimazole (*15*), methimazole (*16*) and methylthiouracil are used to decrease the uptake and concentration of inorganic iodine by the thyroid gland in the treatment of hyperthyroidism. The rapid localisation of iodine in the thyroid gland permits the use of radioactive ^{131}I to treat thyrotoxicosis by inducing local radiation damage and hence impairing thyroid functional capacity[20].

Traces of *fluoride ion* (less than 1 part per million) naturally present in domestic water supplies or added as sodium fluoride or sodium fluosilicate significantly reduce the incidence of dental caries in children by rendering the dentine and enamel of teeth more resistant to acid. This is possibly because of increased amounts of fluorapatite laid down in the teeth. A similar rationale explains the use of sodium fluoride and stannous fluoride in toothpastes. Prolonged intake of larger amounts of fluoride ion leads to mottling of teeth (dental fluorosis) and to bones becoming coarser and denser. High levels of fluoride are biocidal so that sodium fluoride, fluosilicate and fluoraluminate are used in some insecticides, rodenticides and wood preservatives. Treatment of acute fluoride poisoning is by the administration of soluble calcium salts such as the chloride or gluconate to form insoluble calcium fluoride.

Of the remaining halide ions, *chloride* is essential as the major anion in blood serum, but it is so widely distributed in Nature that deficiencies do not arise, except

in association with conditions of salt lack. In this case the use of citrates or other salts for sodium or potassium replenishment can lead to an alkalosis and a continuing loss of alkali cations. Thus, in severe cardiac failure the prescribed potassium salt must be the chloride. *Bromide*, on the other hand, is much less common and is not an essential anion. Formerly, potassium bromide was used as a hypnotic but, because of the wide range of alternative compounds able to produce a nonspecific, reversible depression of the central nervous system, this application is now largely of historical interest.

8. Phosphate Ion

Another physiologically important anion is phosphate which is essential for bone formation, for the buffering of biological fluids and as a component of numerous enzyme systems. Intermediary metabolism and, in particular, carbohydrate utilisation are intricately interwoven with the phosphate cycle through the reversible conversion of inorganic phosphate to high energy phosphate in ATP.

Treatments of diseases such as osteoporosis, rickets and osteomalacia, in which there is a disturbance of phosphate levels, is complicated by the interdependence of calcium metabolism. This topic has recently been discussed in relation to clinical medicine[21]. There is the further difficulty that absorption of phosphate from the bowel can be decreased in the presence of calcium or aluminium salts because of the formation of their insoluble phosphates. Uptake of phosphate by bone is exploited in the treatment of polycythaemia vera by intravenous injection of ^{32}P as sodium phosphate. The resulting irradiation of the neighbouring red bone marrow diminishes the production of red cells.

9. Trace Metal Ions

It is only rarely that excess of one essential metal ion can compensate for deficiency of another. More usually, the effects of a deficiency are exacerbated. Quite generally, excessive intake of essential metal ions is harmful to the organism. This is illustrated by considering the uptake of iron, needed for the synthesis of cytochromes, haemoglobin and a number of enzymes including catalase.

Absorption of *iron* by the individual varies with age, iron status, the amount and chemical form of the iron ingested, and with conditions in the gastrointestinal tract, only about 5–15% of iron in the diet being normally absorbed. Ferrous iron, as the sulphate, gluconate, fumarate or lactate or as ferrous ammonium sulphate, is appreciably taken up into the bloodstream from the duodenum, especially in the presence of ascorbic acid, a reducing agent. Little difference was found in the extent of their absorption between ferrous sulphate and the various chelates, but ferric ammonium citrate or polysaccharide complexes were only very poorly absorbed[22] A ferric hydroxide-dextrin complex is sometimes injected intravenously in cases where oral therapy is impracticable.

Sudden excessive uptake of soluble iron salts, especially by young children, can lead to acute iron poisoning, characterised by the highly corrosive action of iron

salts on the gastrointestinal tract, followed by vomiting, shock, circulatory collapse and coma. Chronic exposure to excessive dietary iron causes iron deposits in many organs and gives rise to haemosideroses and haemochromatoses. Diet-induced examples of the latter occur among the Bantu of South Africa who use iron cooking pots in preparing a carbohydrate-rich gruel.

Minute amounts of *cobalt* are necessary in the diet of herbivores if their alimentary canal microflora are to synthesise enough vitamin B_{12} (cyanocobalamin) for the animals to be maintained in good health. For sheep and cattle a level of 0.1 part cobalt per million (dry weight) of grass is adequate but in parts of Australia, New-Zealand and Scotland this requirement is not met unless the grazing areas are top-dressed regularly with fertilisers containing added cobalt salts. Alternatively, "bullets" of pressed cobalt oxide and iron can be lodged in the reticulorumen of the animal so as to provide a slow, steady source of cobalt ions at a low concentration[23, 24].

Copper deficiency occurs in domestic animals grazing on pasture that is low in copper. Remedial measures include the application of fertilizers containing added copper or the use of suitable salt licks. However, human diets are apparently sufficiently diverse that copper deficiency is not likely in man. Instead, there is the risk that the use of copper cooking utensils and water pipes might lead to an excessive uptake. In conditions where body copper levels are undesirably high it may be necessary to restrict the uptake of copper. Because of the insolubility of copper sulphide, potassium sulphide is sometimes taken orally to render dietary copper unavailable.

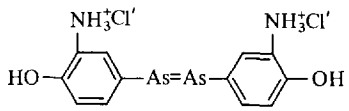
Long-term or intensive corticosteroid or chelation therapy can lead to a sustained decrease in *serum zinc levels,* resulting in delayed healing of wounds and ulcers. Although the mechanism is not known, inclusion of zinc salts in the diet so as to raise blood serum zinc levels to normal also restored healing rates to normal[25, 26]. It is likely that at low zinc concentrations there is impairment of the activity of vital zinc metalloenzymes such as lactic dehydrogenase, alkaline phosphatase, carbonic anhydrase, carboxypeptidase, and of enzymes in which zinc acts as a cofactor. Injection experiments showed that radioactive ^{65}Zn preferentially concentrated in healing tissues[27].

The effects of many different kinds of metal ions are interrelated, both in plants and in animals, so that administration of high levels of one kind of metal ion may lead to deficiency in another. This is usually due to a decrease in the efficiency with which the second metal ion is taken up by the organism. Pastures containing excessive *molybdenum* induce copper deficiency in sheep unless higher than normal levels of copper are supplied. Treatment with high levels of *manganese* induces iron deficiency in soybean plants whereas manganese deficiency develops on high-iron soils. Rats fed zinc-rich diets become copper deficient. Injection of calcium ion is a method of antagonising the effects of excess potassium on the heart and is also used in the treatment of hypermagnesaemia. These examples serve to emphasise the limitations of a simplistic approach to the treatment of metal ion deficiencies and excesses.

IV. Metal-Organic Complexes

1. Arsenicals

The discovery by Ehrlich and co-workers in 1909 of the spirochaeticidal activity of salvarsan (arsphenamine), an organic arsenical, (*17*) led to the development of a wide range of arsenicals for the treatment of syphilis. They owe their effectiveness to their partial conversion to arsenoxides, followed by reaction of the arsenic atom with enzyme -SH groups, particularly of pyruvate oxidase and lipoic acid dehydrogenase, leading to inhibition of cellular metabolism[28]. However, a continuing disadvantage of their use in clinical medicine was their rather low margin of safety so that they have now been superseded in this application by antibiotics such as penicillin.



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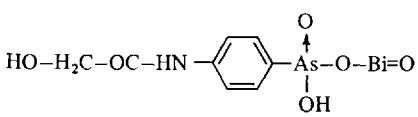

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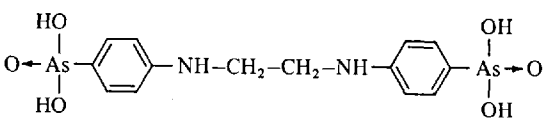

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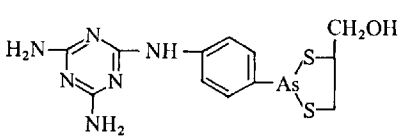

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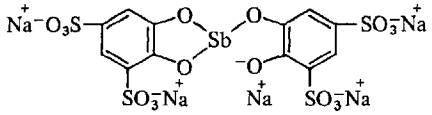
They are still used in the treatment of other parasitic infections. Thus, derivatives of benzene arsonic acid, $C_6H_5AsO(OH)_2$, such as *N*-acetyl-4-hydroxy-*m*-arsanilic acid, *N*-carbamoylarsanilic acid and its oxide, sodium *N*-(carbamoyl-methyl) arsanilate and glycobiarsol (*18*) are potent intestinal amoebicides when taken orally, and difetarsone (*19*) has recently found use as an anthelminthic[29]. They are also administered in the treatment of trypanosomiasis and helminthiasis in animals, as well as in topical application to destroy *Trichomonas vaginalis* and monilinia. For the first of these purposes they have been largely replaced by melarsoprol



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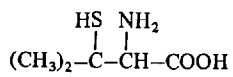
(*20*) (which is of interest because its arsenic atom is already chelated to two sulphur atoms) and by non-arsenical trypanocides.

Inorganic arsenicals find limited use as pesticides. Examples include arsenious oxide in cattle dips, zinc arsenite as a wood preservative, and calcium and lead arsenates and Paris green (double salt of copper arsenite and copper acetate) as insecticides[30].

2. Antimonials

Organic antimonials behave similarly to the arsenicals but their mode of action is believed to be inhibition of phosphofructokinase activity of the parasite[31]. Although their high toxicity has been a disadvantage in human applications, antimony sodium or potassium tartrate, sodium antimonyl gluconate, stibophen (*21*) and sodium α,α′-dimercapto-succinate, injected intravenously or intramuscularly, are used to control schistosomiasis, filariasis and leishmaniasis. Longer retention times in the body are achieved when the antimony is injected as antimony dextran glycoside[32].



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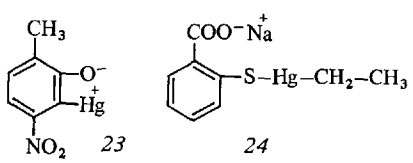
Formation of a mixed chelate of antimony sodium tartrate with penicillamine (*22*) yields a nontoxic material suitable for intramuscular injection, that retains its antiparasitic action in schistosomiasis[33]. A similar detoxification of organic arsenicals was observed in the presence of penicillamine. Penicillamine also protects against liver damage by antimony sodium tartrate[34].

3. Mercurials

Another group possessing activity against -SH groups in enzymes are the organic mercurials such as mercurochrome, nitromersol (*23*), phenylmercuric nitrate and



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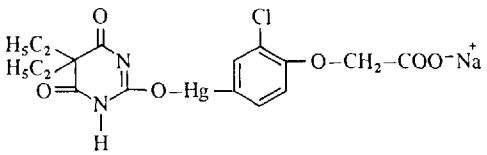

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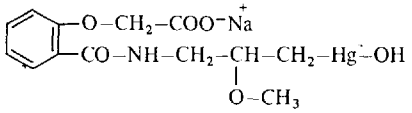

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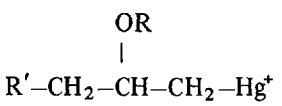
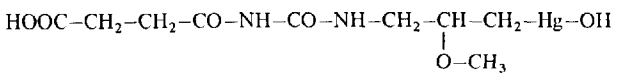
acetate, and thimerosal (*24*). They are bacteriostatic antiseptics. The organic mercurial merbaphen (novasurol) (*25*) introduced as an anti-syphilitic agent, proved to be a good diuretic but to be undesirably toxic. Subsequently, mersalyl (salyrgan) (*26*) and many other organic mercurials have been proposed as diuretic agents. Most of them are substituted mercuriisopropanols,



where R is alkyl (commonly methyl) and R′ is widely variable but usually contains a hydrophilic group[35]. Examples of organic mercurials used for intramuscular injection include meralluride (mercuhydrin) (*27*), mercurophylline (mercupurin) (*28*) and mercaptomerin (thiomerin) (*29*). Their main application is in the treatment of cardiac oedema.



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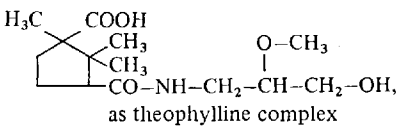

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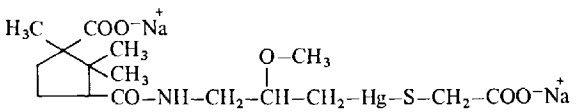

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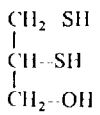
They act on the kidney by depressing the mechanisms that govern the active reabsorption of sodium and chloride ions. They are rapidly excreted by the kidney but their use is hazardous because their action is believed to be due to inorganic mercury ions released by rupture of the carbon-to-mercury bond, probably followed by the firm attachment of the mercury ion to a sulphydryl group of a renal enzyme. The administration of dimercaprol (*30*), a strong chelating agent for mercury, removes mercury from the kidney and terminates the diuretic action. It is of interest that Paracelsus used calomel (mercurous chloride) as a diuretic.



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Organic mercurials still find extensive application as fungicides for the disinfection and protection of grain seeds against fungal attack[36]. This use has decreased somewhat following alarm at the environmental consequences arising from the widespread distribution of such toxic materials.

4. Other Metals

Although their method of operation is not understood, injections of *gold chelates* such as aurothioglucose, sodium aurothiomalate and aurothioglycanide (α-auromercaptoacetanilide) provide symptomatic relief in active rheumatoid arthritis[37]. Careful supervision is required to prevent the development of haematological crises. Attempts have been made to synthesise organo-gold complexes that are active when taken orally[38, 39]. A different application of gold therapy in rheumatoid arthritis has been the injection of colloidal radioactive ^{198}Au into the knees of badly affected patients to bring about radiation synovectomies. The underlying principle is that the colloidal particles are localised on the synovial membranes nonselectively, and this is the site of the resulting radiation damage[40]. Other, more suitable, β-ray emitters that are now used in this way are colloidal ^{186}Re, ^{90}Y and ^{169}Er.[40]. A former widespread application of gold salts, now of only historical interest, was in the treatment of tuberculosis.

Triphenyl- and tributyltin compounds have strongly biocidal properties and are employed in some agricultural fungicides and wood preservatives[42]. Other possible applications include disinfectants for use in hospitals and for the eradication of snails that transmit bilharzia. However, these compounds are potentially hazardous because of the ease with which they inhibit oxidative phosphorylation in man. Oral administration of diethyltin diiodide as a treatment for boils led to many deaths[43].

Cyanocobalamin (vitamin B_{12}), a cobalt complex first isolated from liver but now produced commercially from microbiological culture, is needed to maintain normal synthesis of red blood cells in man and animals. Ruminants obtain cyanocobalamin from their symbiotic rumen flora while in other herbivores such as the

rabbit and the chicken it is synthesized in the caecum and colon, but man depends for his daily requirement of around 0.1–1 μg on meat and other animal products. Its major medical application is, by injection, in the prophylaxis and treatment of pernicious anaemia and other macrocytic anaemias.

V. Chelation Therapy

Animal and plant tissues contain many different complex-forming species which compete for the essential metal ions that are also present. Among the more important species and metal-binding groups are amino acids, peptides, carboxylic acids, phosphates and the mercapto, dithio, amino, imidazole, phenolic and free carboxyl groups of proteins. The resulting complex formation can have many effects. These include the masking of active centres in the ligand, changes in electron distribution, the imposition of a particular stereochemistry on the ligand, and changes in the liposolubility of the ligand, modifying the ease with which it, and the metal ion, can penetrate cell membranes. Complex formation can also alter the oxidation-reduction potential of a metal ion.

Enhanced complex stability is observed when a metal ion is bound by two or more groups on the same ligand to form five- or six-membered chelate rings. The factors that govern the stability of such complexes have been discussed elsewhere[44], but it should be noted that the common donor atoms in complex-forming species are oxygen, nitrogen and sulphur. Metal ions of low ionic charge and rich in *d*-electrons [such as Cu(I), Cu(II), Hg(I), Hg(II), Zn] bind more strongly to nitrogen and sulphur-containing ligands in the sequence S > N > O : higher valent cations and alkaline earth cations form their most stable complexes with anionic oxygen ligands such as carboxylate and phosphate. The stabilities of metal complexes usually lie in the sequence

$$\text{Cu(II)} > \text{Ni} > \text{Co(II)} \sim \text{Zn} \sim \text{Cd} > \text{Fe(II)} > \text{Mn(II)} > \text{Ca}.$$

Because the toxic effects of heavy metal ions are due to their strong complexing ability towards reactive groups that are essential for normal physiological function, the usual treatment in cases of heavy metal poisoning is to administer a more powerful chelating agent which removes, competitively, the toxic cations. For maximum effectiveness, especially where the toxic metal ion can be deposited in the bone, treatment should be started as soon as possible.

Differences in the distribution of toxic metal ions within the body are often found when *acute and chronic poisoning* are compared. Whereas chronic poisoning frequently results in deposition in particular tissues such as lead and strontium in bone, or excessive copper in liver, brain and kidney, acute poisoning is usually accompanied by excessive concentrations of the metal ion in the gastrointestinal tract, the blood and the soft tissues. These distribution patterns are important when treatment with a chelating agent is required, particularly if there is any danger that the metal chelate might be translocated to a more vital area.

Excessive levels of essential metal ions can be undesirable and chelation therapy may be needed for adequate control to be achieved. The treatment of patients suffering from Wilson's disease (hepatolenticular degeneration in which there is intracellular deposition of copper in the liver and brain, accompanied by a deficiency of the copper-containing protein, caeruloplasmin) is an example[45].

Chelating agents for use in removing metal ions have to meet a number of requirements. They must be watersoluble, be resistant to metabolic degradation and retain adequate chelating ability at physiological pH values. Their molecular size and structure must permit them to penetrate to sites where metal ions are bound or stored, and they must react rapidly and relatively specifically with the metal ions that are to be removed. The agents and their metal chelates must be readily excreted by the kidney and they must not be appreciably toxic, either as the free chelating agents or as their metal complexes, at therapeutic concentrations. Preferably, the chelating agent should be active when given by mouth. It is necessary to take account of the effects of chelating agents on other, essential, metal ions that are also present in biological systems.

Administration of high levels of chelating agents during pregnancy may be potentially hazardous. Rats fed a diet which contained 3% disodium EDTA produced many malformed young unless the diet also contained 0.1% zinc ion[45]. It has been suggested [46,47] that strong chelating agents are teratogenic because of their ability to deprive enzymes of the metal ions essential for their activity. Processes that may be especially vulnerable are collagen synthesis and maturation, possibly because of their dependence on copper ions. Attribution of their reported teratogenic effects to chelation[47] is rather doubtful for cortisone, salicylates and diphenylhydantoin because they are probably less powerful chelating agents than some of the amino acids and other metabolites that are ordinarily present. High intakes of salicylates did not increase mineral excretion by pregnant rats, nor did it decrease mineral uptake by their foetuses[48].

With the development of computers with large "memories" it has been possible to construct models to calculate equilibrium concentrations of all species in multimetal – multi-ligand systems simulating physiological conditions, using published stability constant data[49, 50]. This approach should permit the assessment of how effective an added chelating agent is likely to be in competition with ligands already present in a biological situation. One application confirmed the clinical observation that penicillamine and triethylenetetramine can be used to deplete blood copper levels in sufferers from Wilson's disease[50]. A continuing difficulty, however, is to allow quantitatively for the possibility of mixed-ligand metalcomplexes being formed. For a review of the formation and stability of mixed complexes, see Ref.[51].

1. Dimercaptopropanol (*30*)

The first chelating agent developed as an antidote to a heavy metal poison was 2,3-dimercaptopropanol (dimercaprol, British Anti-Lewisite, BAL). Originally intended for use on victims of the arsenical vesicant poison gas Lewisite[52], it has since proved efficacious in the treatment of antimony, gold and mercury poisoning as well as

becoming the standard material for use in arsenical poisoning. It has been largely replaced by penicillamine for copper chelation in the treatment of Wilson's disease. Early work on the chemistry, biochemical properties and clinical applications of dimercaptopropanol is described in Refs.[52] and [53].

The -SH groups of dimercaptopropanol react with heavy metal ions including arsenic, to form very stable five-membered chelate rings, displacing heavy metal ions that would otherwise bind to essential -SH groups of enzymes such as succinoxidase and pyruvic oxidase. In this way, most of the enzyme activity can be restored if therapy is commenced soon after exposure.

However, dimercaptopropanol is not ideal for chelation therapy. It is unstable in aqueous solution and is easily oxidised so has to be injected intramuscularly as a solution in vegetable oil. This ensures its slow release into body tissues, making for more effective action, but it has a most objectionable odour. The injections are painful and frequently give rise to unpleasant local reactions.

Because of the ease with which dimercaptopropanol can be broken down in the body there is a danger that chelation, followed by breakdown, will simply result in the translocation of the metal ions to other tissues such as brain or liver. High doses of dimercaptopropanol can adversely affect a number of essential metal-activated enzymes, such as catalase, carbonic anhydrase and peroxidase, and also produce dangerous systemic effects. Dimercaptopropanol cannot be used to remove cadmium because its cadmium complex is toxic to kidney tissue[54].

Related sulphur-type ligands that have been suggested for use in treating heavy metal ion poisoning include disodium dimercaptosuccinate[55, 56], dihydrothioctic acid[57], sodium 2,3-dimercaptopropane sulphonate (unithiol)[58], potassium methyl- and ethylxanthates[59], and sodium diethyldithiocarbamate[60]. Unfortunately, many potentially useful dithiols are too toxic for clinical use.

2. EDTA

Ethylenediamine tetraacetic acid (EDTA) was introduced originally as a water-softener and as a textile dyeing assistant because of its ability to form very stable, water soluble complexes with many metal ions, including calcium and magnesium. The equilibria involved in chelation of metal ions by EDTA and related ligands have been exhaustively studied, notably by G. Schwarzenbach and his colleagues, and provide the basis for complexometric methods of chemical analysis. EDTA and its metal complexes have also become probably the most familiar examples of agents used in chelation therapy.

Rapid intravenous injection of the sodium salt of EDTA depletes blood calcium levels and produces hypocalcaemic tetany, but, carefully administered, this agent can be used to treat hypercalcaemia and to overcome digitalis-induced arrhythmia by adjusting the Ca^{2+}/K^{+} balance[61]. The ready availability of calcium ion from extracirculatory stores enables slow (drip) infusion of sodium EDTA without untoward effects. Continued over several days, calcium is mobilised from bone and calcium EDTA is excreted in the urine but plasma calcium levels are not greatly affected[62]. Although it has been little explored, drip infusion of sodium EDTA,

accompanied by an antiatherogenic diet, might be usefully employed for the attempted decalcification of atherosclerotic plaques as an aid to their regression[63–66]. A related application has been the use of irrigation with EDTA, in buffered solutions, for the dissolution of stones containing calcium oxalate, carbonate or phosphate, lodged in the urinary tract[67–69]. Formation of soluble calcium chelates is also the principle involved in the use for a similar purpose of renacidin, a commercial preparation containing derivatives of citric, malonic and gluconic acids.

Because it chelates calcium ion needed for the conversion of prothrombin to thrombin, EDTA is a good anticoagulant for the storage of blood.

Injection of calcium disodium EDTA does not affect blood calcium levels but heavy metal ions in the body have a higher affinity for EDTA than does calcium, and hence these metals readily exchange *in vivo* to form soluble EDTA-heavy-metal complexes that are excreted in the urine. Because of its poor absorption from the alimentary tract, calcium disodium EDTA is usually injected intramuscularly or intravenously. These considerations led to its being used, initially in 1952[70], to treat lead encephalopathy.

Lead is widely destributed in the environment, especially in industrial and urban areas, and it is readily absorbed into the mammalian body where it exerts a number of undesirable physiological effects. Its most dramatic action is the inhibition of human red cell δ-aminolaevulinic acid dehydrase activity[71], but it also depresses the activities of many enzymes having functionsl -SH groups. Attempts to remove lead from the body using agents such as dimercaptopropanol can result in the formation of lipid-soluble lead complexes that may be carried to the brain and exacerbate the effects of lead poisoning.

This danger is minimal if calcium disodium EDTA is used as the chelating agent and it has now become the material of choice in cases of lead poisoning. Except in massive doses it is almost nontoxic and treatment with $CaNa_2EDTA$ results in a rapid depletion of lead in the soft tissues: prolonged treatment is necessary for the removal of lead from bone[72].

EDTA is a powerful chelating agent but has poor selectivity so that its use results in the excretion in the urine of an appreciable fraction of the body's zinc reserves. These need to be restored by supplementation. Its general chelating ability suggests that $CaNa_2EDTA$ might be of use in treating other heavy toxicities. However, it is unsuitable for removing mercury from the body because the considerable extent to which mercury ions are hydrolysed in solution partly offsets the complexing ability of EDTA and also because mercuric ions bind more strongly to sulphur-type ligands such as enzyme -SH groups.

Based on the avidity of cobalt for cyanide ions, intravenous injection of the cobalt EDTA complex has been recommended as being the best antidote in cyanide poisoning[73]. Earlier therapy was based on sodium nitrite and sodium thiosulphate, with partial conversion of haemoglobin to methaemoglobin.

Compounds related to EDTA have been examined for use in chelation therapy but appear to offer little additional advantage except for diethylenetriaminepentaacetic acid which is currently the most effective agent for removing ^{239}Pu from the body[74]. A difficulty in using chelating agents to remove radioactive elements such

as plutonium, thorium and uranium is the ease with which these metal ions become deposited on, and incorporated into, bone.

The highly polar character of aminopolycarboxylic acid chelating agents such as EDTA and DTPA can be a disadvantage in their use to eliminate toxic metal ions from the deeper parts of the body and from inside cells. This is because these chelating agents disappear rapidly from the body and they also fail to penetrate cell membranes. The effectiveness and penetration persistance of these agents might be improved by using analogues in which one or more of the hydrogens attached to the carbon backbone was replaced by a large alkyl or some other suitable lipophilic group. An alternative, and promising, approach is to encapsulate the chelating agent in a liposome made of phosphatidylcholine and cholesterol[75].

EDTA has also been used therapeutically in agriculture to treat iron deficiency in plants, particularly fruit trees, on basic soils. Spraying with iron-EDTA solution facilitates absorption of iron into the plant through its roots, followed by breakdown of the iron chelate so that the iron becomes available to the plant[76].

3. D-Penicillamine (*22*)

D-Penicillamine, β,β-dimethylcysteine, has proved to be a good chelating agent for promoting the excretion of copper in patients with Wilson's disease and for prolonged use in controlling this disease[77, 78]. Advantages over dimercaptopropanol include water-solubility and stability, so that it can be given by mouth. In the rare cases where hypersensitivity reactions develop, triethylenetetramine (*31*) is a suitable alternative[79]. Penicillamine is also useful as a chelating agent for mercury and

$$NH_2-CH_2-CH_2-NH-CH_2-CH_2-NH-CH_2-CH_2-NH_2$$

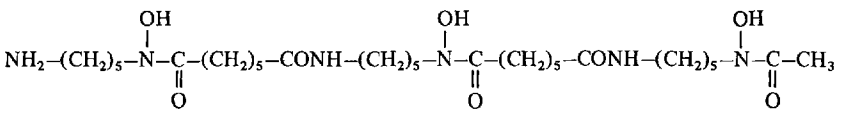
31

lead[80, 81], although N-acetylpenicillamine is reported to be more effective in treating mercury poisoning[81, 82]. After an initial series of injections of calcium disodium EDTA, it is more convenient to use oral administration of penicillamine for the prolonged treatment of lead poisoning.

It is doubtful whether the therapeutic application fo penicillamine in rheumatoid arthritis[83] is related to its metal-chelating ability. Metal ion depletion undoubtedly occurs and it has also been observed that patients on protracted penicillamine therapy had decreased taste sensitivity; this was restored to normal when oral copper was given[84].

4. Desferrioxamine (*32*)

Desferrioxamine B (Desferal), a chelating agent that occurs naturally in certain microorganisms, has a very high affinity for ferric ion but only a relatively weak affinity for most other cations. Under physiological conditions it can complex free iron and competitively remove iron from ferritin and haemosiderin. It also binds,



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to some extent, transport-iron but not iron in porphyrins or attached to transferrin[85, 86]. Desferrioxamine is readily soluble in water but is poorly absorbed from the gut.

These properties have made this material useful for the treatment of acute iron poisoning[87, 88]. Desferrioxamine is administered partly by mouth, to prevent further uptake of iron, and partly by slow intravenous drip or by muscular injection so that iron already absorbed will be complexed and excreted in the urine. Desferrioxamine also finds limited use in the therapy of diseases such as haemochromatosis and haemosiderosis which are associated with excessive iron storage in the tissues.

5. Others

Other chelating agents have had limited trials in metal ion toxicities. For example, sodium diethyldithiocarbamate has been used to treat nickel carbonyl[89] and thallium[90] poisoning but dangerous redistribution of thallium has been observed when this treatment was used. Results suggested that thallium was transported as the lipid-soluble chelate across the blood-brain barrier and deposited in the brain where it produced characteristic neurological symptoms[91].

The development of chelating agents for use in metal ion removal is still in its infancy and suitable antidotes are not yet available for treating poisoning by beryllium, cadmium, manganese, thallium and many other toxic metal ions. Radiostrontium and radium pose particular problems because of their chemical similarities to calcium.

The clinical uses of metal-binding drugs have been reviewed[92, 93]. Reference[94] gives a general account of metal-induced toxicities and chelation therapy.

VI. Antibacterial Action of Chelating Agents

Divalent cations are important in the maintenance of cell structure of Gram-negative bacilli. Chelation of these cations with EDTA leads to major disorganization of the cell surface of organisms such as *Escherichia coli* or *Pseudomonas aeruginosa.* This decreases their resistance to antibacterial agents, including polymyxin B, benzalkonium chloride and chlorhexidine diacetate[95], which depend for their action on penetration into the cell. Conversely, the bacteriocidal effect on these bacilli is decreased if calcium or magnesium ions are added to strengthen cell surfaces[95, 96].

In this way, by reducing permeability barriers, combination therapy with antibiotic and chelating agent may enhance significantly the efficacy of suitable antibiotics in the topical treatment of local infection.

8-Hydroxyquinoline and its water-soluble 5-sulphonate are used in dermatology to treat fungal and bacterial infections. Its action on Gram-positive bacteria is due to the formation of lethal 1 : 1 chelates with suitable metal ions, including copper(II), iron(II) and iron(III), that are present in the cell or the medium[2]. A limitation to the use of 8-hydroxyquinoline and its derivatives is their inhibition by red blood cells. Closely related compounds, similarly used in dermatology, include halquinol (5-chloro- and 5,7-dichloro-8-hydroxyquinoline) and chlorquinaldol (5,7-dichloro-2-methyl-8-hydroxyquinoline). 5,7-Diiodo- and 5-chlor-7-iodo-8-hydroxyquinoline have been applied in treating amoebiasis. The latter (vioform) is used against bacterial dysentery but, since 1970, has been banned in Japan because of undesirable side-effects.

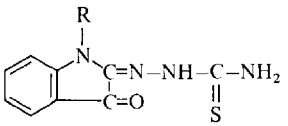
1,10-Phenanthroline and a number of its derivatives were lethal to dermatophytes and *Candida albicans* when tested as hydrochlorides, methiodides and metal chelates[97]. Substituents in the 1,10-phenanthroline molecule did not greatly change the antimicrobial activity except that *Candida albicans* was more sensitive to chelates of the 5-phenyl or 5-methyl derivatives. The labile manganese(II), cadmium, and copper(II) chelates were the most, and the methiodides were the least, potent. It is suggested that whereas metal ions usually penetrate poorly into a microbial cell the formation of a metal chelate may facilitate metal ion uptake from the medium in which the cell is growing, leading to toxicity. Alternatively, diffusion of the chelating agent into the cell may lead to a critical depletion of essential metal ions in the cell. Metal [Fe(II), Cu(II), Cd, Ni, Ru(II)] chelates of 1,10-phenanthroline bases are lethal to cultured mammalian cells at concentrations down to 0.1 μM[98]. This cytotoxicity limits their possible medical uses to topical applications.

Similar, but somewhat less, activity was shown by 2,2′-bipyridine bases and their metal chelates[99].

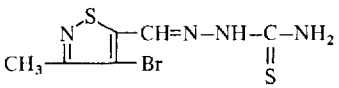
Because free zinc, cobalt or manganese ions are necessary for the toxic action of *Clostridium perfringens*, calcium EDTA (which is a strong chelating agent for these ions) was active as a therapeutic and detoxifying agent for gas gangrene in mice and guinea pigs[100]. Subsequently, calcium disodium diethylenetriaminepentaacetate was found to be 10–20 times as effective, possibly because it binds zinc more strongly and possibly because it is retained longer in the body[101].

VII. Antiviral Action of Chelating Agents

Although virus replication comprises numerous biochemical transformations that might provide suitable targets for antiviral therapy, up to the present time the applications of chelating agents in this field have been largely empirical. In 1950, Hamre *et al.*[102] found that benzaldehyde thiosemicarbazone protected mice against vaccinia infection. Later the thiosemicarbazones of nicotinaldehyde, isonicotinaldehyde, 2-thenylaldehyde and 3-thenylaldehyde proved to be more active[103]. Following the demonstration that isatin 3-thiosemicarbazone (*33*) had greater antiviral activity



33 R=H
34 R=CH_3



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against vaccinia virus[103], further derivatives having yet greater activities were synthesised[104]. One of these, methisazone (1-methylisatin 3-thiosemicarbazone) (*34*) was used successfully in clinical trials as prophylaxis against smallpox[105]. This was the first time that a drug had been shown to be effective in the prevention of a virus disease in man. Likewise, 4-bromo-3-methylisothiazole-5-carboxaldehyde thiosemicarbazone (*35*) afforded substantial protection against smallpox when it was administered in a controlled prophylactic trial but was ineffective once the disease was established[106].

Methisazone has also been shown to be active against tumour-causing viruses[107], adenovirus[108), 109] and alastrim[110]. It is useful for treating complications following vaccination[111].

The antiviral activity of methisazone and related thiosemicarbazones affects a late stage of the pox virus growth cycle[112] by interfering with viral mRNA translation[113]. It is likely that this antiviral activity is due to the ability to form terdentate chelates with essential heavy metal ions by bonding through a sulphur, a nitrogen and either an oxygen or a nitrogen atom. A similar structure-activity relation has been suggested to explain the anticancer effects of a number of related compounds[114]. The effect of methisazone on adenovirus is abolished if the sulphur is replaced by oxygen[115]. However, interpretation of the physiological effects of substitution in the thiosemicarbazones is rendered more difficult by variations in their limited solubilities and in their partition coefficients between oil and water.

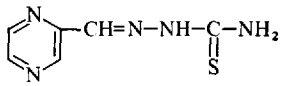
For the control of most virus diseases, vaccination remains the treatment of choice so that likely applications of chelating agents as antiviral agents are limited, especially as many of the common virus infections are comparatively trivial. One possible field would be as a prophylactic against influenza viruses because they mutate sufficiently frequently that it is difficult to provide effective vaccines. It has recently been shown that the RNA-dependent RNA polymerase activity of influenza viruses is a zinc-containing enzyme which can be inhibited *in vitro* by a number of chelating agents including bathocuproine, bathophenanthroline and heterocyclic thiosemicarbazones[116]. In this way, multiplication of influenza virus can be prevented. Currently, the effects of calcium disodium EDTA and calcium trisodium diethylenetriamine pentaacetate, used to deplete body zinc levels and limit the amounts of zinc available for incorporation in enzyme systems, are being studied[117].

Certain scrapie-like diseases of sheep in Iceland and some chronic encephalopathies in man (including kuru, the "laughing disease" of New Guinea) are the result of slow virus infections with long incubation periods. 1-Methylisatin 3-thiosemicarbazone has been found to reduce the reverse transcriptase activity and cytopathic effect of the RNA slow viruses producing the sheep diseases and hence may provide a chemotherapeutic method for their control[118].

VIII. Anticancer Action of Chelating Agents

Regression of tumours in mice has followed the ingestion of the chelating agent L-penicillamine[119]. Also, it has been observed that several families of cytostatic agents are able to chelate metal ions. These facts, and the dependence of many enzymes on the presence of trace metal ions, lead to the logical suggestion[120] that new anticancer drugs could be looked for among chelating agents. One of the most fruitful classes of compounds belonging to this group that have been examined are heterocyclic thiosemicarbazones.

Although too toxic for clinical use, 2-formylpyridine thiosemicarbazone is mildly antileukaemic[121]. The 3- and 4-formyl isomers are inactive. Based on the premise that two requirements for activity were a nitrogen heterocyclic ring system and atoms so placed that terdentate N – N – S chelation was possible with a metal ion[114] many thiosemicarbazones derived from compounds with a carbonyl group in a position α to a heteroaromatic nitrogen atom have been synthesised. A number of these were active against experimental neoplasms. For example, introduction of a 3-hydroxy group into the pyridine portion decreased its toxicity and increased its carcinostatic activity[122] while the 3-(*m*-aminobenzyloxy)-derivative was active against an ascites tumour in mice but was not toxic[123]. 2-Formyl-5-hydroxypyridine thiosemicarbazone, injected intraperitoneally into mice, gave protection against leukaemia and several other types of cancer[124]. 1-Formylisoquinoline thiosemicarbazone[125] and many of its 5-substituted derivatives[126] also show antitumour activity. 2-Formylpyrazine thiosemicarbazone (*36*) was more active, and less toxic, than 2-formylpyridine thiosemicarbazone[114].



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Recent work suggests that these and related α-(*N*)-formylheteroaromatic thiosemicarbazones may act by inhibiting tumour-derived ribonucleoside diphosphate reductase[127], possibly by chelating iron which is an essential cofactor in this enzyme system. The resulting decrease in the availability of deoxyribonucleotides inhibits the synthesis of RNA. Replacement of the sulphur atom in 5-hydroxy-2-formylpyridine thiosemicarbazone by selenium led to retention of activity against ascites cells and to inhibition of DNA synthesis. The corresponding nitrogen and oxygen analogues were inactive[128] as, also, was salicylaldehyde thiosemicarbazone[129].

Some bis(thiosemicarbazones) have antitumour activity. Thus 2-keto-3-ethoxybutyraldehyde bis(thiosemicarbazone) has marked carcinostatic and carcinolytic activity against several established rat tumours[130] while the bis(N-methylthiosemicarbazones) of hydroxymethylglyoxal, methylglyoxal and ethoxymethylglyoxal were inhibitors to a sarcoma in mice[131].

6-Formylpurine thiosemicarbazone is a potent inhibitor of ribonucleoside diphosphate reductase and is active against herpes simplex and cytomegalovirus[132], but is nephrotoxic. Attempts, so far unsuccessful, have been made to prepare derivatives which possess an antitumour effect but are less toxic[133].

Clinical studies with 5-hydroxy-2-formylpyridine thiosemicarbazone[134, 135] were not very promising. No antitumour effects were noted in patients with solid tumours and only transient improvement was observed in some of the patients with acute leukaemia. Dose level was limited by gastrointestinal toxicity, and activity was shortlived. The sparing solubility in water of many of the thiosemicarbazones is a continuing limitation to their use in cancer therapy but the retention of activity of 2-formylpyridine thiosemicarbazone when a 3-(*m*-aminobenzyloxy) group was inserted into the molecule suggests that it should be feasible to attach a polar group to such a substituent to increase water-solubility without loss of potency[123].

Hydroxymethylglyoxal bis(guanylhydrazone), also a powerful chelating agent, actively inhibited an adenocarcinoma in mice and was antileukaemic[136].

IX. Antifungal Action of Chelating Agents

The earliest fungicides used on plants were sulphur and inorganic copper compounds, and the latter still find major agricultural use, current examples being copper oxychloride and Bordeaux mixture. They act by forming a surface layer on plants which inhibits the growth of any fungal spores that alight on it. These fungicides do not penetrate into the plant tissue and are ineffective once infection occurs[36]. The copper 8-hydroxyquinoline complex, used extensively to rotproof canvas, also finds a limited application as an antifungal agricultural spray.

More recently, a range of organic fungicides have been developed, including a number based on dithiocarbamic acid. These comprise preparations such as zineb and maneb, respectively the zinc and manganese ethylenebisdithiocarbamate chelates, and ziram and ferbam (zinc and ferric dimethyldithiocarbamates)[30]. They owe their activity to the ability of copper(II) ion present in the environment or the plant to displace these metals, with the formation of trace quantities of the sparingly soluble 1 : 1 copper chelates[137]. The effectiveness of the fungicide tetramethylthiuram disulphide is due to its slow reduction under field conditions to dimethyldithiocarbamate.

In human applications, bis(pyridine-2-thiol-1-oxidato)zinc chelate has bacteriocidal and fungicidal properties comparable with selenium sulphide but is less toxic to mammals, making it suitable for controlling dandruff and seborrhoeic dermatitis[138].

X. Robust Metal Complexes as Antibacterial Agents

In aqueous solution, the complexes of most metal ions exist in dynamic equilibrium with their components. However, especially where metal ions have 3, 4 (low-spin), 5, or 6 *d* electrons and where complex formation involves large values of ligand field stabilisation energy, "inert" or "robust" complexes may be formed. Such complexes persist unchanged for hours or days under conditions favourable to their dissolution, and their biological properties are often strikingly different from those of their components.

Robust metal chelates from several transition metal ions with 1,10-phenanthroline and related bases have been shown to be lethal to many bacteria and pathogenic fungi. When applied topically they are of low toxicity to patients and the microorganisms fail to become resistant. Thus, the chemically inert tris(1,10-phenanthroline) complexes of nickel and ruthenium(II) are bacteriostatic to a number of typical Gram-negative and Gram-positive microorganisms and *Mycobacterium tuberculosis*[99]. These chelates are unsuitable for injection into animals because of their powerful neuromuscular blocking action.

They have been used prophylactically and therapeutically in the treatment of topical infections due to *Staphylococcus aureus* and other bacteria and fungi. In clinical trials, the nickel chelate of 3,4,7,8-tetramethyl-1,10-phenanthroline was effective against staphylococcal infection in the newborn, in patients undergoing obstetric surgery and in the treatment of adolescents infected with acne vulgaris[139]. The corresponding copper(II) chelate controlled monilial infection of the nail and soft tissues, and was effective in chronic monilial and trichomonal vaginitis[139]. The usefulness of the manganese(II) complex was also assessed[140].

The ion $[PtCl_6]^{2-}$ is bacteriocidal to *E. coli* whereas *cis*$[PtCl_4(NH_3)_2]$ inhibits cell division and produces filamentous growth[141]. Complexes of the type *trans*-$[RhL_4X_2]Y$, where L is substituted pyridine, X is chloride or bromide and Y is chloride, bromide, nitrate or perchlorate, had high levels of antibacterial activity against Gram-positive organisms and *E. coli*[142].

XI. Robust Metal Complexes as Anticancer Agents

Administration of copper dimethylglyoxime[143], copper 2-keto-3-ethoxybutyraldehyde bis(thiosemicabazone)[144] or copper pyruvaldehyde bis(thiosemicarbazone)[145] to rodents has led to inhibition of tumour growth. The copper(II) complex of 2-keto-3-ethoxybutyraldehyde bis(thiosemicarbazone) transports copper into neoplastic cells where it is deposited and inhibits a number of enzymes responsible for the synthesis of DNA[146].

In 1969 several inorganic platinum metal compounds, including the *cis*-dichlorodiamminoplatinum(II) and *cis*-tetrachlorodiamminoplatinum(IV) complexes were shown to have antitumour activity[147] very similar to the action of bifunctional alkylating agents such as nitrogen mustard. The latter, like the platinum complexes, also cause filamentous growth in *E. coli.* The platinum complexes are effective against a number of animal neoplasms, including mammary tumours induced by a carcinogen[148], the Ehrlich ascites carcinoma and leukaemias[149], by selectively inhibiting their deoxyribonucleic acid synthesis[150]. However, the possible dose levels are limited by their nephrotoxic and immunosuppressive properties[151]. A qualified usefulness of *cis*-dichlorodiamminoplatinum(II) as a broad spectrum anticancer agent has been established in clinical trials[152–155]. It has been suggested[156] that better antitumour activity with less toxic effect might be obtained clinically by prolonged infusion of low doses of the agent. The *cis*-dichloro-(dipyridine)platinum(II) complex is less active but is appreciably less toxic[157]. The corresponding *trans* com-

plexes are inactive. Dichloro(4,5-dimethyl-*o*-phenylenediamine-N,N')platinum(II), *in vitro*, inhibited DNA, RNA and protein synthesis and decreased the viability of tumour cells more rapidly than did the chloroplatinum ammine[158].

Binding sites for the platinum complexes in the DNA molecule are believed to be the amino groups of adenosines and cytidines, with the formation of a bidentate chelate ring by the *cis* platinum complexes[159].

XII. Miscellaneous

1. Applications Based on Selective Binding

Advantage can sometimes be taken of selective binding of inorganic species and chelating agents to achieve a desired objective in particular tissues. Thus, ^{67}Ga, as gallium citrate, has been used in locating malignant neoplasms by a scanning technique, based on the affinity of gallium for such tissues[160, 161]. This observation may have clinical application in assessing the therapeutic benefit of chemotherapeutic and radiotherapeutic treatments. There is the additional advantage that gallium salts show some antitimour activity against sarcomas[162]. A new approach to the treatment of tumours is suggested to be based on the incorporation of ^{10}B into antibodies and their subsequent binding into neoplastic tissues. The resulting irradiation by a source of slow neutrons leads to nuclear disintegration of the ^{10}B and to the emission of shortrange α-particles which destroy the malignant tissue[163].

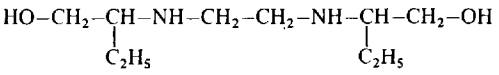
Not all chelation is advantageous. For example, the strong metal-binding ability of the antibacterial tetracyclines causes difficulties in their use in medicine. Their effectiveness is diminished if they are administered orally in the presence of materials, such as alumina gels, iron preparations or milk, which contain metal ions that are readily chelated. The structures of the tetracyclines enable them to be absorbed and bonded strongly to the surface of hydroxyapatite[164] so that patients medicated with tetracyclines develop fluorescent yellow bands in zones of actively growing bone or teeth and there can be a temporary inhibition of bone growth[165].

Conversely, deposition of a chelating agent on the surface of teeth may sometimes be advantageous. Thus, dental caries and corrosion are claimed to be prevented by exposing teeth to calcium sugar phosphate[166]. The resulting deposit apparently inhibits subsequent chelation and dissolution of calcium that normally occurs in teeth as a result of the oxidation of carbohydrate particles to polyhydroxylic acids.

2. Examples where Chelation and Activity may be Unrelated

Although many therapeutic agents have metal-binding properties, it is not always clear whether or not this is fortuitous. *p*-Aminosalicylic acid is a chelating agent but acts as a metabolic analogue of *p*-aminobenzoic acid which does not chelate metal ions. Other antitubercular drugs that are also chelating agents include isoniazid (isonicotinic acid hydrazide), thiacetazone (*p*-acetamidobenzaldehyde thiosemicarba-

zone) and ethambutal (*37*). However, according to calculations using a blood plasma model for a mixture of copper ions, zinc ions and amino acids[50], chelation is unlikely to be an important factor in the physiological effects of isoniazid. Thiosemicarbazones of benzaldehyde and some of its derivatives, active against tubercle bacilli[167], are unlikely to be effective chelating agents in a biological environment whereas the clinically successful antiviral drug methisazone probably owes its activity to its ability to chelate metal ions. The antihypertensive drug hydralazine (*38*) is a strong chelating agent but this is probably not the reason for its activity[168]



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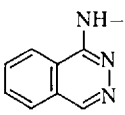

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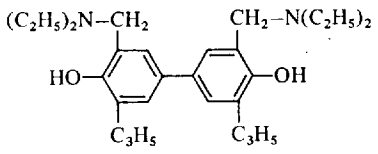

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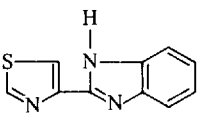

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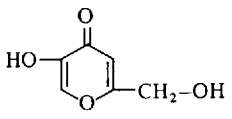

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It is more difficult to decide whether chelation plays any part in the activity of salicylic acid which is released in the biodegradation of aspirin and, especially in cases of abuse of this drug and in the therapy of rheumatic disease, may be present in appreciable quantities in the body.

The observation that aminothiols protect living organisms against ionizing radiation has been explained on the basis of their chelating ability for copper ions[169]. This is consistent with the requirement that the amino and the thiol groups are separated by not more than three carbon atoms, that the molecule contains a free thiol group (thio-ethers are inactive), and that *N*-alkylation decreases, but does not abolish, activity, However, alternative explanations have been proposed, including the scavenging of free radicals and the possibility that aminothiols bind to, and stabilize, parts of the DNA helix, thereby diminishing radiation damage and facilitating repair[170].

Ohter drugs in which their chelating ability has not yet been shown to be essential include 5-nitrofurfuraldehyde semicarbazone (an antibacterial applied locally to wounds, burns and skin infections), dichlorophene (2,2′-methylene-bis-[4-chlorophenol] used as a germicide and against tapeworms), biallylamicol (*39*) (used in amoebiasis), thiabendazole (*40*) (a vermicide[171], and the antibiotics kojic acid[172] (*41*), antimycin[173] and bacitracin[174]. Bacitracin A is more stable when it is in the form of its 1 : 1 zinc chelate[175]. The antimicrobial activity of bacitracin is stimulated

by zinc ions and is inhibited by chelating agents such as EDTA[176]. Phleomycin was isolated as a water-soluble copper complex, but removal of the copper had very little effect on its antibiotic activity[177]. Again, although the anthracycline drugs daunomycin and adriamycin form stable chelates with many cations this produces no significant change in their antitumour activity[178].

Insulin probably exists in the pancreas as a zinc compound, but administration of insulin, *per se*, is effective in controlling diabetes. The main reason for using a zinc-containing preparation such as neutral protamine zinc insulin is that it is stable, dissolves more slowly and lasts longer than insulin, itself, when given by hypodermic injection.

Many agents that reduce blood pressure in man have metal-complexing ability, including sodium azide, sodium thiocyanate, dimercaptopropanol, hydralazine and, possibly, sodium nitroprusside.

3. Some Untoward Effects of Chelating Agents

Hexachlorophene, (2,2′-methylene-bis-[2,4,6-trichlorophenol]), widely used as a germicidal agent against Gram-positive cocci, is a strong chelating agent which sometimes produces defects in the central nervous system of premature infants. These lesions are similar to those observed with the copper-binding substances cuprizone and isonicotinic acid hydrazide[179]. The related germicide bithional, (2,2-thiabis-[4,6-dichlorophenol]), although it is also a chelating agent, does not have this effect and is suggested as an alternative[179].

Sodium diethyldithiocarbamate, dimethyldithiocarbamate and disulphuram are potent inhibitors of the enzyme dopamine-β-hydroxylase of brain, possibly because of chelation of copper. The copper-diethyldithiocarbamate complex penetrates the central nervous system more readily than does copper ion, itself. These effects slow the disappearance of pentobarbitone from plasma and produce behavioural changes in rats[180].

Some metal binding substances, including 8-hydroxyquinoline and sodium dimethyldithiocarbamate, damage the islets of Langerhans and cause diabetes in experimental animals[181].

Large cations have a powerful effect on neuromuscular transmission, resulting from acetylcholine esterase inhibition and curariform activity. Examples are quaternary nitrogen, phosphorus and arsenic bases and chelate species such as the tris-(2,2′-bipyridine)iron(II) cation when it is injected intravenously. Bis(2,2′,2″-terpyridine)ruthenium(II) perchlorate has about one tenth of the activity of curare[182].

4. Macrocyclic Ligands

It seems likely that the future will see major developments in the use of metal complexes as bacteriocidal, viricidal, immunosuppressive, anti-arthritic and biocidal agents. One class of complexing agents that appears to have particular promise are the macrocyclic antibiotics, macrocyclic polyethers, polyamines and "cryptates". In this group the ligand wraps around a metal ion to form a lipid-soluble complex

in which the metal ion is effectively "hidden" from the solvent. This gives rise to the interesting situation that although the complexes may contain an alkali or an alkaline earth metal ion, their properties are determined by their predominantly hydrophobic exteriors, so that these complexes dissolve in nonpolar solvents or pass through biological membranes[183]. Because of this behaviour, such ligands are also known as ionophores. For these reasons, and also because the size of the cavity in the interior of the ring structure makes for selective cation binding, such complexing agents can be expected to have important biological applications in reactions and equilibria involving metal ions.

Naturally occurring examples include the antibiotics valinomycin (a cyclododecadepsipeptide), enniatins (cyclohexadepsipeptides), macrotetrolides such as nonactin and monactin, and the polyether, polyalcohol, monocarboxylic acids monensin, nigericin and dianemycin. Most of these fungal metabolites bind potassium much more strongly than they bind sodium ion and exert biological effects such as the uncoupling of oxidative phosphorylation and the induction of adenosinetriphosphatase which are related to the way in which they influence transport of sodium and potassium ions across cell membranes[185]. Valinomycin selectively increases the permeability of potassium ion across natural membranes[185] so that, in anaerobic bacteria, the excessive potassium ion transport leads to loss of potassium ion from the cell[185] and inhibits protein synthesis[186]. This appears to be the cause of its bacteriostatic action. The addition of more potassium ion to the medium can reverse the inhibitory effect of the antibiotic on these microorganisms[187]. By contrast, inhibition of growth of *Bacillus subtilis* by valinomycin was greater in the presence of potassium ion, possibly reflecting increased dissipation of metabolic energy[187].

Most of the ionophores elaborated by microbes are extremely toxic to animals and hence are of only limited use as antibiotics. More importantly, however, knowledge of their mode of operation raises the possibility of synthesising physiologically useful ligands with diminished toxicity. Many macrocyclic polyethers (so-called "crown compounds"), polyamines, polythioethers and related compounds able to function as cryptates have been synthesised[188, 189]. Replacing oxygen atoms by sulphur or nitrogen atoms greatly reduces the complexing ability for alkali and alkaline earth metals, while enhancing it for heavy metal ions, so that macrocycles bonding through sulphur and nitrogen atoms form complexes with transition metal ions but not with alkali metals. A synthetic bicyclic cryptate, with a much greater affinity for strontium than for calcium has been used to remove ^{85}Sr from rats[190].

5. Silicones

Because of their chemical inertness, low surface tension and antisurfactant activity, polyorganosiloxane (silicone) oils have found varied applications in medicine. These have included use as an artificial lubricant for arthritic joints[191], as a means of soft tissue augmentation, and as an additive in creams and oils for burn treatment. Dimethylpolysiloxane fluids have been injected as replacement for aqueous and vitreous humour in eyes. Reactive organosilicon compounds comprise a new class of potential prophylactic and therapeutic agents[192]. Protection against atherosclerosis

in rabbits on an atherogenic diet, effects on the nervous system, and activity against bacteria and fungi are some of the reported effects of organosilicon compounds. Others are insecticides, insect repellants and herbicides[192]. Because the polyphenylcarbamoylalkyl silicon compounds absorb ultraviolet light, they have been proposed for use as sunscreen agents in skin creams.

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