Perspective

No Life on this Planet Without PHB

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Dedicated to the memory of my dear friend and admired colleague *Jack D. Dunitz*

The history of discovery by *Rosetta Reusch* of oligo- and poly-β-hydroxybutyrates (OHBs and PHBs) consisting of less than *ca.* 150 HB units is described. These 'short-chain' biopolymers can be detected in all living organisms and have numerous physiological activities of fundamental importance for the chemistry of life. The largest are components of ion channels such as Ca^{2+}-polyphosphate-PHB (Ca-PPi-PHB) in genetically competent *E. coli* and in mammalian mitochondria. Sequences with chain lengths $< ca.$ 30 occur covalently attached to proteins (post-translational PHBylation), and methyl esters of the dimer and trimer are used by certain bacteria as highly efficient antioxidants. With synthetic monodisperse OHBs (up to 128mer) our group has contributed structural investigations, and we have shown that OHBs ≥ 16 alone make phospholipid bilayer vesicles permeable to Ca ions. An extensive biochemical analysis of the TRPM8 protein channel, responsible for the sense of heat in our skin, proved to be fully active only when PHBylated. Reasons for the difficulty of detecting OHBs and PHBs are discussed: the polyester chain is highly flexible, and there is ester cleavage by base, acid, nucleophiles, *Lewis* acids, and heat – in stark contrast to peptides. PHBs may be called a ubiquitous but *fleeting* species in the chemistry of life – worth being appreciated and studied much more intensively in the future! A speculation about PHB's possible role in prebiotic compartmentalization is presented, and recent uses of compartmentalization in organic synthesis are briefly mentioned.

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Keywords: compartmentalization, history of science, ion channels, non-proteinaceous ion channels, organic synthesis in water, PHBylated proteins, poly-β-hydroxybutyrates, polyphosphates, prebiotic chemistry, TRPM8 channel.

1. Brief Overview of PHB History

In apparent contrast to the statement of the title, the average natural scientist (chemist, biochemist, medicinal chemist, with the possible exception of the material scientist) looks up in surprise when asked about the biopolymer poly-β-hydroxy-butanoic acid (PHB; *Table 1*). However, the general practitioner, the clinician, indeed every premedical student, will know

that the monomer, β-OHB, is a major component of so-called *ketone bodies*, together with acetyl-acetate (AcAc) and acetone (CH_3COCH_3). Ketone bodies are formed from fatty acids by oxidative degradation in the liver, especially when there is a shortage of carbohydrates: starvation, excessive exercise, carbohydrate-free diet, deficiency of insulin. See the terms ketosis, acidosis, ketone stress, acetone breath as well as recent review articles.[1–4]

The polymer PHB was discovered by *M. Lemoigne* almost 100 years ago.[5] It is a biopolymer produced by microorganisms as a storage material under conditions

of nutrient limitation in the presence of an excess carbon and energy source (certain cyanobacteria can use CO_2 and light, resp.!)[6−8] PHB and other poly-(β-hydroxy-alkanoic)-acids, PHAs) of high molecular weight ($>10^3$ units) are deposited in cells in the form of granules (up to 90% of the cellular dry weight) and can be produced biotechnologically on large scale. PHAs have excellent material properties (*Figure 1*) and are fully biodegradable. Thus, numerous groups worldwide have investigated PHAs; for a personal selection of prominent scientists in this field and for some leading review articles that have appeared in the past 20 years see *Figure 2* and references.[9−14]

Parallel to the material-driven investigations, *Rosetta Reusch* – widely unnoticed, poorly recognized and almost single-handedly – discovered and studied the physiological properties of different kinds of PHB. Starting in the early 1980s, she identified PHB of chain lengths in the range of 100 to 200 HB units as components of ion channels in cell membranes, where it is associated with polyphosphate (PPi), another orphan biopolymer (*Table 1*).[15−23] Furthermore, *Reusch* found that PHB of chain lengths below *ca.* 30 units occurs as an appendage to proteins, formed by post-translational PHBylation. *Reusch*'s contributions up to the year 2005 were summarized in an article by *V. Norris* (*Figure 3*). An extensive review published in 2012[24] by *Reusch* was entitled 'Physiological Importance of Poly-(*R*)-3-hydroxybutyrates', and a complete list of her publications is attached as *Supplementary Material S1*.

In the meantime, very short oligomers of 3-hydroxy-butanoic acid have been identified as physiologically active compounds. Thus, the methyl esters of the HB dimer and trimer are used by bacteria against oxidative stress caused by reactive oxygen species RO• (ROS), with higher activities than the common antioxidants glutathione and vitamin C.[25−27] In a previous report, the HB tetramer was characterized as a growth promoting factor in certain microorganisms.[28]

2. Entry and Contributions to the PHB Field by Our Group

The entry of our group into PHB chemistry took place at a time when we were demonstrating the usefulness of readily available natural products as chiral building blocks for syntheses of enantiomerically pure complex target molecules.[29−32] An example was our synthesis of elaiophylin, which required access to (*R*)-3-hydroxy-butanoic acid (*Figure 4*);[33−36]1 we had found that the (*S*)-form is accessible *via* reduction of ethyl acetoacetate with fermenting baker's yeast.[38,39] A literature search for the (*R*)-enantiomer led to PHB, to its biotechnological production by *ICI* in Billingham UK, to a contact with Dr. *Atkins* of the *ICI* (*Figure 1*), and to our *Organic Syntheses* procedure for large-scale degradation of PHB to (*R*)-3-hydroxy-butanoic acid and its methyl ester.[40,41] Eventually, the engagement with PHB led to the discovery in our group of the non-natural β-peptides – from a world *without* hydrogen bonds to a world *with* hydrogen bonds![42]2 (*Figure 4*).

Dieter Seebach was born in Karlsruhe, Germany, in 1937 and studied chemistry at the local Technische Hochschule (now KIT), where he received a PhD degree in 1964 with a thesis on small-ring compounds and per-oxides (supervisor R. Criegee). After a two-year stay at Harvard University as Postdoctoral Fellow (with E. J. Corey) and as Lecturer he returned to Karlsruhe for a Habilitation (1969) on S- and Se-stabilized carbanion and carbene derivatives. In 1971 he became Full Professor at the Justus Liebig Universität Giessen and in 1977 he moved to ETH Zurich. He held longer-term guest professorships, for instance at the University of Wisconsin (Madison), at Caltech (Pasadena), and at Harvard University. Main areas of research in the past: reactivity umpolung, pool of chiral building blocks, structure of Li-compounds, chiral dendrimers, TADDOLs, self-regeneration of stereogenic centers, S-, Se-, Si-, NO_2-, F-, Ti-organic compounds, PHB, and β-peptides. From 2004, the year Seebach became Professor emeritus at ETH, until 2014, he did research with a group of postdoctoral co-workers on the mechanism of organocatalysis and on biological investigations of β-peptides and of cyanophycin derivatives. This work was financed by the Swiss National Science Foundation and by Novartis. Without funding, laboratory space, or external collaborations Seebach now enjoys discussions with coworkers of active groups in the LOC, with colleagues worldwide, and with former group members, and he takes more time with his family and for achieving an acceptable state of health.

1For a more recent use of β-hydroxy-butanoate see ref. [37].

2For the original German version of this article see *Supplementary Material S2* as pdf-file.

Table 1. Well known and hitherto less well appreciated biopolymers.

Monomers	Oligomers	Biopolymers
Metabolism	Regulation, recognition, signaling	Catalysis, storage, structure, information
Amino acids	Oligopeptide	Polypeptides (enzymes, silk, wool)
Carbohydrates	Oligosaccharides (glycans, blood-group det.)	Polysaccharides (cellulose, starch, chitin)
Acetic acid, isoprene derivatives	Isoprenoids (steroids)	Polyisoprenoids (rubber, gutta-percha)
Nucleotides, Nucleosides	Oligonucleotides (t-RNA)	Polynucleotides (DNA)
β-Hydroxy-alkanoic acids; 3-HB is major ketone body	OHBs (PTM PHBylation) (HO• scavenger) (growth-prom. factor)	Poly(β-hydroxy-alkanoates) PHAs (storage PHB) (ion-channel component) (*cf.* polymalate, PMA)
Phosphoric acid	Oligophosphates (ADP, ATP, CoA)	Polyphosphate, PPi[a] (energy storage) (ion-channel component)
Phenolic styrenes made from Phe and Tyr		Lignin (mechanical support and water transport in plants)

[a] For brevity PPi is used for 'inorganic polyphosphate' throughout this paper.

Courtesy of J. Atkins (ICI, UK) and H. Brandl (University Zürich)

Figure 1. Granulate of PHB from the ICI biotechnological process, articles made from BIOPOL (PHB/PHV-copolymer), demonstration of biodegradability and Cautionary Tales. https://www.scotchem.ac.uk/wp-content/uploads/2019/02/Biopol-IBiolC-compressed.pdf.

HELVETICA
chimica acta

M. Lemoigne   S. J. Park

G.-Q. Chen   O. P. Peoples

E. A. Dawes   Y. Poirier

Y. Doi   H. G. Schlegel

P. A. Holmes   A. J. Sinskey

D. Jendrossek   A. Steinbüchel

R. H. Marchessault   B. Witholt

Figure 2. *M. Lemoigne*, the discoverer[5] of PHB, and a selection of prominent researchers in the area of PHAs.

"These scholars who talk to the wind": Introductory comments
Y. Thomas, P. Mentré, Cellular and Molecular Biology **2005**, *51*, 579-582.
DOI 10.1170/T666

Poly-(R)-3-hydroxybutyrate and the pioneering work of Rosetta Natoli Reusch
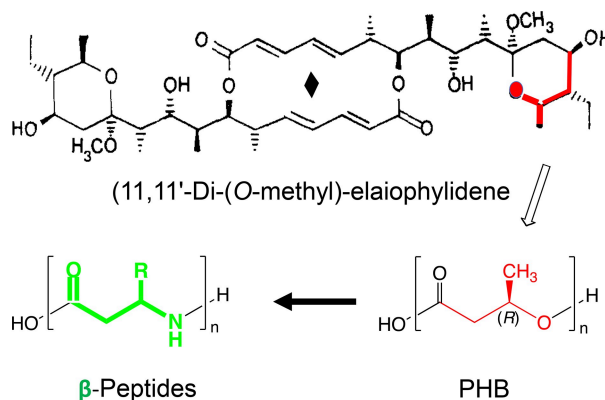V. Norris, Cellular and Molecular Biology **2005**, *51*, 629-634.
DOI 10.1170/T673

GENETIC COMPETENCE
CALCIUM CHANNELS
CALCIUM PUMPS
POTASSIUM CHANNELS
DNA CHANNELS
POST-TRANSLATIONAL MODIFICATIONS BY PHB
MEDICAL SIGNIFICANCE
ORIGINS OF LIFE

Figure 3. Special issue of *Cell. Mol. Biol.*, and the article by *V. Norris* therein, covering the work of *R. N. Reusch* up to 2005; the subtitles in this review are given in capital-letter print. DOI 10.1170/T673

(11,11'-Di-(*O*-methyl)-elaiophylidene)

β-Peptides   PHB

Figure 4. Synthesis of an enantiomerically pure elaiophylin derivative using (*R*)-β-hydroxy-butanoic acid from PHB as a building block.[33–37] For an extensive review article on β-peptides see ref. [41].

MICROORGANISMS
E. coli
baker's yeast
ANIMALS
human serum albumin, aorta tissue
bovine serum albumin, brain, lung, heart, liver
porcine liver, heart
sheep intestine
cat muscle
snail hump
**PLANTS**
spinach leaves
gorse
peanuts
beet stem
spinach

	Rat normal		Rat diabetic
PHB [μg/g]	(±15%)	PHB [μg/g]
12	Plasma	71
3	Sciatic Nerve	30
9	Kidney	67
10	Aorta	43
6	Eye	19
4	Brain	5

Figure 5. PHB detected in various kingdoms of life[43] and enrichment of PHB in diabetic *vs.* healthy rats.[44] Courtesy of *Rosetta Reusch.*

Next, we became involved in improving analytical methods for detecting PHB, especially by NMR spectroscopy.[43] Our group members were busy collecting samples in the supermarket, in the bakery, in the butcher's shop, in the pet shop, and in the university hospital. The fact is that PHB was detected in every living organism or part thereof, and *R. Reusch* found that its concentration depends upon the state of health of animals (for instance with or without diabetes; *Figure 5*).[43,44] The most sensitive method of detection is due to *Rosetta Reusch*: the use of an anti-PHB F(ab')$_2$, an antibody[45,46] with detection limits in the picogram range for short-chain (< *ca.* 30) PHB in covalently PHBylated proteins. For a survey of the PHB

analytics available at the turn of the century see *Figure 12* in our review article entitled 'Detection, synthesis, structure, and function of oligo(3-hydroxy-alkanoates): contributions by synthetic organic chemists'.[47]

As synthetic organic chemists, we could not resist the temptation to prepare various monodisperse oligomers of β-hydroxy-butanoic acid (OHBs) and study their properties. Thus, we noticed that heating PHB or poly(3HB-co-3HV) under transesterifying conditions and distillation *in vacuo* led to cyclic oligomers as thermodynamic products, with the triolide being preferred (*Figure 6*).[48–50] Crystallization of triolides under various conditions provided a clear demonstra-
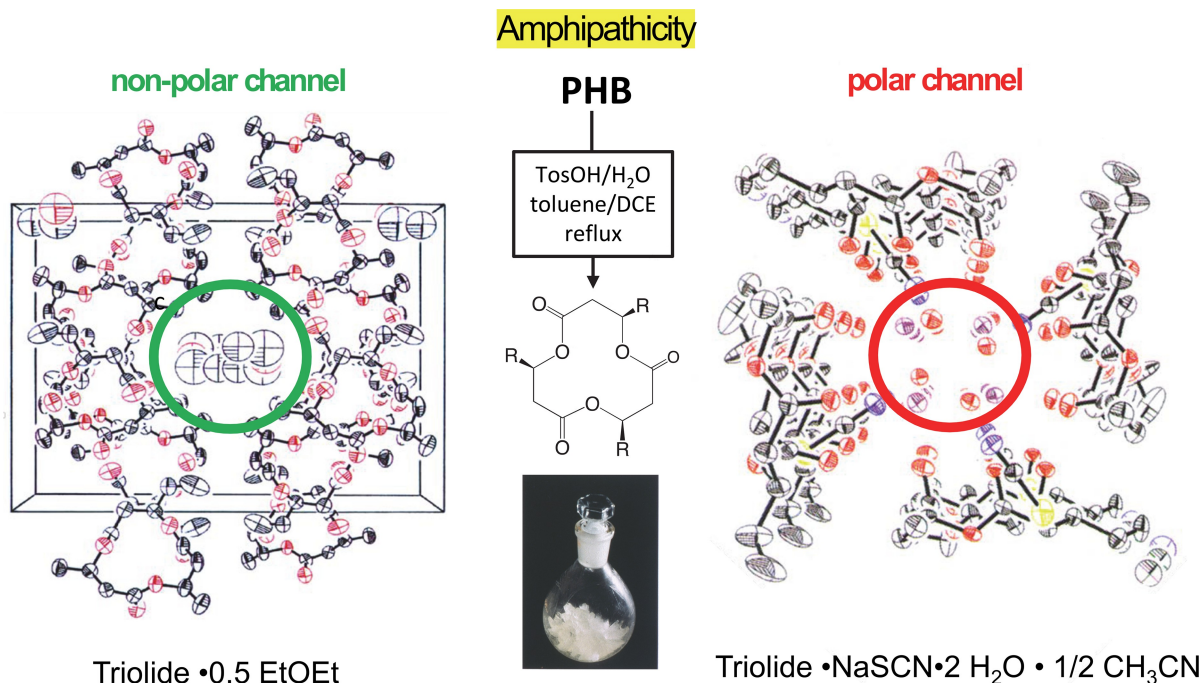
Figure 6. Preferred triolide formation from PHB (or PHV) and crystal structures of inclusion complexes of triolides with diethylether and with NaSCN. Methyl groups point inside the channel housing Et₂O. Carbonyl oxygens point inside the channel housing hydrated Na^+ ions, with the NCS^- anions sticking to the wall through their sulfur atoms.[48–50] Reproduced with permission from Reference [50], Copyright 1994, *Wiley-VHCA*, Switzerland.

tion of PHB's amphipathicity: see the crystal structures of inclusion compounds with the non-polar diethyl ether and with the thiocyanide sodium salt in *Figure 6*. Using fragment coupling, we synthesized monodisperse linear OHBs up to 128 units long. We also prepared oligolides of up to 128-atom ring sizes, coumarin-labeled oligomers, bicyclic and dendritic derivatives of β-hydroxybutanoic acid, and we investigated malic-acid analogs (*Figure 7*).[51–60]

A preferred folding mode for PHB in the solid state was revealed when we treated a stirred suspension of PHB in THF at −75 °C with LiN(SiMe₃)₂ (Li-HMDS): The resulting mixture contained an increasing amount of 15-mer (by size-exclusion chromatography), indicating that the polymer chain is folded in crystalline domains, that the cleavage by Li-HMDS occurs at the folding points, and that *ca.*15 HB units span the thickness of the crystallites.[61,62] For the 2₁-helical PHB structure, which has a *ca.* 6 Å pitch,[49] the crystallite would be *ca.* 48 Å thick. This was confirmed in a collaboration with *Peter Barham*'s group in Bristol (UK):[62–66] Crystallites of OHBs of various chain lengths, and of the polymer were prepared and investigated by electron diffraction, electron microscopy, wide and small angle X-ray diffraction, and atomic force microscopy (*Fig-*

ure 8,**A** and *8*,**B**). Our linear OHBs consisting of 16, 32, and 96 HB units, as well as PHB itself, all form crystallites of *ca.* 50 Å thickness, indicating that the chains of ≥ 32 HB units must be folded.

In view of the role PHB plays in cellular ion channels (*vide supra*), we tested the ability of OHBs to transport ions between two aqueous phases through a lipophilic environment, first in a simple U-tube experiment (*Figure 8*,**C**).[67] As a bulk liquid organic membrane we chose methylene chloride (density 1.33 g/cm³) containing a linear HB 28mer or various cyclic OHBs, as counter anion in the aqueous phase we used the yellow picrate, and we tested the transport rates of alkali (Li to Cs) and alkaline-earth (Mg to Ba) ions. Result: all cations were transported and all OHBs were found to be transporting ionophores, without special selectivities, except that the largest ions Cs^+, Ba^{2+} (which have smaller heats of solvation in water) were fastest – and Li^+ slowest.

We then turned to the patch-clamp technique, in which a POPC phospholipid bilayer (PLB) is placed in an aperture in such a way that a voltage can be applied between the two sides of the bilayer. Preparation of bilayers in the presence of 0.1 to 5 % of a linear OHB consisting of n = 4, 8, 16, 32, 64, 96 HB units led
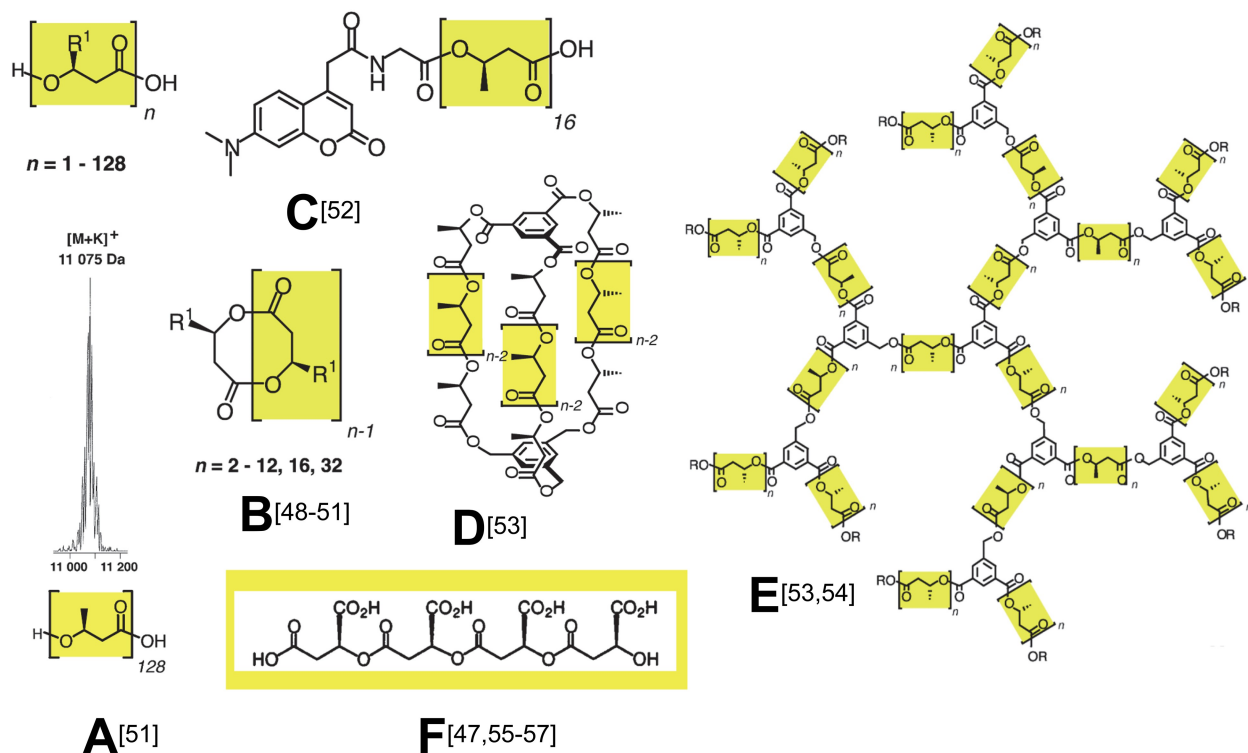
Figure 7. Synthesized OHB and oligomalate derivatives. **A**: Linear OHBs consisting of 4, 8, 16, 32, 64 and 128 HB units. **B**: Oligolides of β-hydroxybutanoic and hydroxypentanoic acid containing up to 32 building blocks. **C**: A fluorescence labeled OHB. **D**: A bicyclic compound, in which trimesic acid and benzene-1,3,5-trimethanol function as bridgeheads, and three OHBs as the bridges. **E**: A dendrimer with OHBs between the branching points. **F**: A linear oligomer of malic acid; various other malates were synthesized; the anionic (*cf.* DNA, RNA, PPi) biopolymer PMA has interesting physiological functions in certain microorganisms.[58–60]

to voltage-driven conductance for Rb^+ and Ba^{2+} ions with $n \geq 16$ (*Figure 8*,**E**, collaboration with *R. N. Reusch*).[68] Moving even closer to cellular systems (*cf.* cell walls, liposomes, microsomes), permeation of Ca^{2+} ions through the PLB of unilamellar vesicles encapsulating the Ca-complexing Quin-2 dye was studied. Indeed, when linear 32- or 64-mer OHB had been incorporated into the vesicle wall it became Ca^{2+} permeable! (*Figure 8*,**D**, collaboration with *P. Walde*, ETH Zürich).[69]

We have thus clearly demonstrated that simply incorporating OHBs into phospholipid bilayers renders them ion permeable! The amphipathicity (*cf. Figure 6*) of OHBs is evident: they have an affinity for the hydrophobic region of the bilayer, and, once inside, can transport hydrophilic ions through the bilayer. There are two intriguing questions: *1)* is the matching thickness of *ca.* 50 Å of OHBs/PHB and standard phospholipid bilayers accidental or evolutionary?, and *2)* is the chain length of ≤ 30 HB units in posttranslationally modified PHBylated proteins correlated with the folding propensity of the HB chain in this chain-

length range (*vide infra*)? For examples of PHBylated proteins, including the *E. coli* outer membrane protein A (OmpA) see the corresponding chapters in the article presented in *Figure 3* and in *Reusch*'s review article.[24]

3. The Non-Proteinaceous Calcium/Polyphosphate/Polyhydroxybutyrate (Ca-PPi-PHB) Channel

When *E. coli* cells are incubated in calcium salt solutions, they become genetically competent, *i.e.*, their cell wall becomes permeable to DNA (*transformation*).[70–72] This process is performed numerous times every day worldwide in molecular biology laboratories, for instance to express large amounts of proteins or enzymes, or to perform site-directed mutagenesis. If asked how this transformation occurs, the average molecular biologist will shrug and admit ignorance – it works! I have done this test many times. The matter-of-fact answer is: *R. Reusch* showed
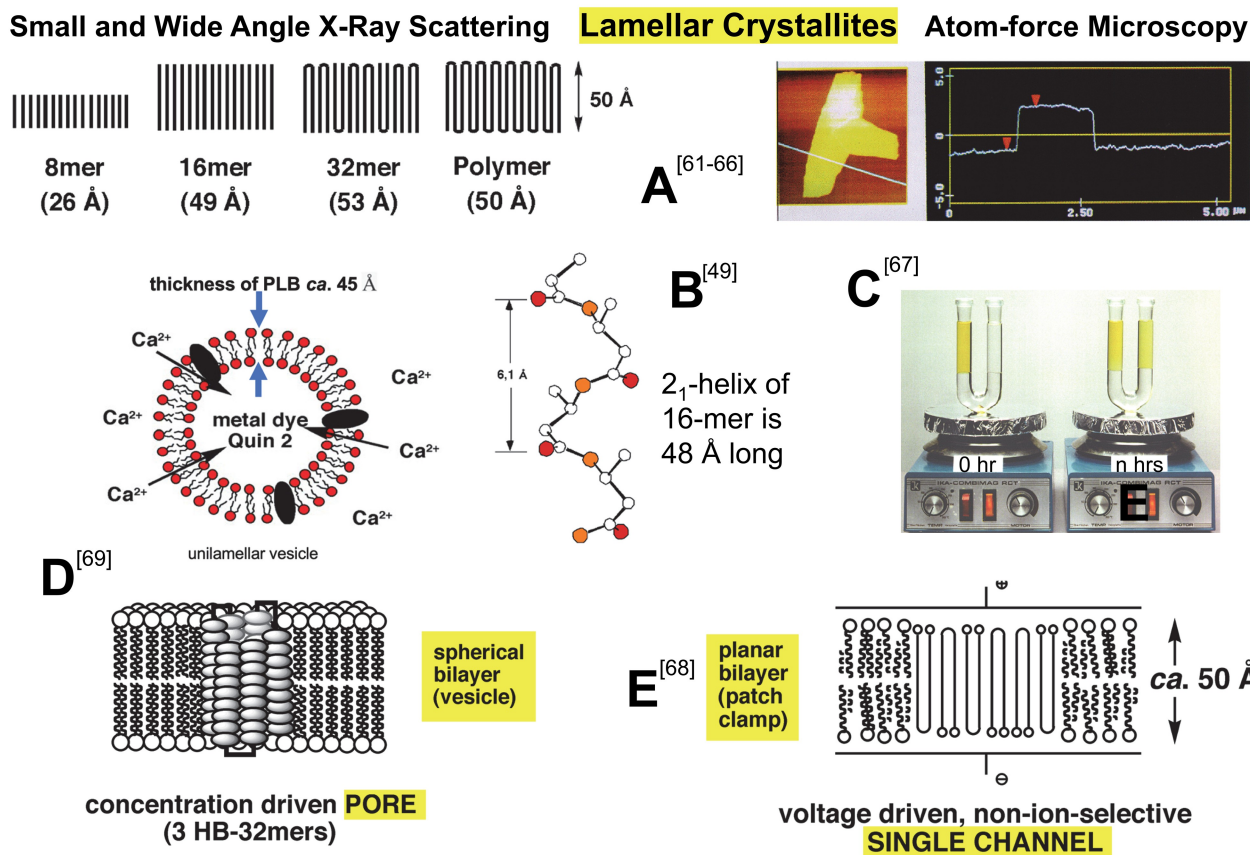
Figure 8. Lamellar crystallites of linear OHBs and of PHB (**A**), 2_1-helix of PHB chain (**B**) and ion transport through hydrophobic phases by OHBs as ionophores (**C**, **D**, **E**). For both, the spherical (**D**) and the planar (**E**) bilayer preparation palmitoyl-oleoyl-phosphatidylcholin (POPC) was used, a common phospholipid in eukaryotic cell membranes.

in 1988 that *PHB is involved*,[73] the Ca^{2+} ions induce the microorganism to form an ion channel consisting of calcium polyphosphate (PPi, *ca.* 60 residues) and PHB (*ca.* 135 residues). This non-proteinaceous channel can be extracted from *E. coli* and incorporated into PLBs for patch clamp experiments,[73–75] which show selectivity for divalent (Ca^{2+}!) over monovalent cations. The same channel (with somewhat longer polymer chains) has been isolated from rat liver mitochondria by water-free chloroform extraction.[76–78] To make sure that no additional but undetected component is present in the natural Ca-PPi-PHB channel from *E. coli* cells and from mitochondria we synthesized a complex from our 128mer OHB and Ca(polyP), the latter being obtained from sodium polyphosphate glass (Av residue number 65, *Sigma–Aldrich*) and calcium chloride. The resulting complex was incorporated in a POPC (*Avanti Polar Lipids*) bilayer and used for patch-clamp experiments. Result: the potential-current dependence of the synthetic channel is superimposable with that of the channel extracted from *E. coli* (*Figure 9*).[79] Other properties of the natural and the synthetic Ca-PPi-PHB channel, such as ion selectivity, gating characteristics, or temperature effects (only up to 40 °C!)[80] are also identical.

The discovery that the Ca-PPi-PHB channel can also be extracted from mitochondria is particularly important in view of the fact that these organelles are not only the 'powerhouses' (ATP synthesis) in eukaryotic cells but also function as 'storehouses' for calcium; there is constant Ca^{2+} pumping between the inside and outside of mitochondria through the inner (IMM) and outer mitochondrial membranes (OMM)[21,81–84] – and obviously PHB is involved![76–78] For further physiologically important functions of mitochondria see textbooks of biochemistry, and for leading references about inorganic polyphosphate in physiology *vide supra*,[15–23] see especially the leading contributions by *A. Kornberg*.[15–17]
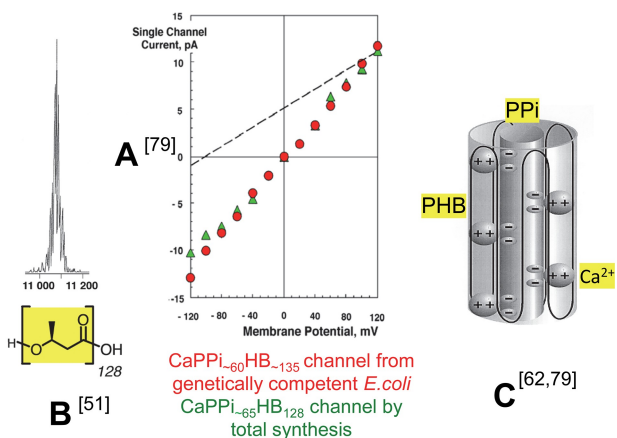
Figure 9. A: Conductance for Ca^{2+} of the non-proteinaceous voltage-activated calcium/polyphosphate/poly-β-hydroxybutyrate (Ca-PPi-PHB) channel (patch-clamp experiment); red circles: channel from *E.coli*, green triangles: synthetic channel. **B**: Synthetic monodisperse HB 128mer for preparation of the (Ca-PPi-PHB) channel. **C**: A proposed structure of the (Ca-PPi-PHB) channel.

4. The TRPM8 Channel: Cold-Temperature Sensor in the Peripheral Nervous System, PHB in Our Daily Lifes, and Problems of Detecting PHB

The 2021 *Nobel* Prize in Physiology and Medicine was awarded jointly to *David Julius* (University of California, San Francisco) and *Ardem Patapoutian* (Scripps Research Institute) for 'their discoveries of the receptors for temperature and touch' (https://www.nobelprize.org/uploads/2021/10/press-medicineprize2021.pdf).

The laureates and their groups identified receptors in the mammalian peripheral nervous system that sense heat, cold, pressure, as well as compounds producing the sensation of burning (capsaicin of chili pepper) or of cold (menthol, icilin). These receptors are members of the **T**ransient **R**eceptor **P**otential ion channel family present in the plasma membranes of animals and gated by phosphatidylinositol 4,5-bisphosphate [PI-(4,5)P$_2$].[85] One of the best studied TRP channel proteins is TRPM8,[85–94] and here we go: it is a calcium channel, it is modulated by inorganic polyphosphate,[87] it is regulated by PHB,[88] and it is post-translationally modified (PTM) by PHBylation![89,90] An exhaustive biochemical analysis of the TRPM8 protein by state-of-the-art methodologies was published in 2013 by the groups of *E. Zakharian*, *E. Pavlov*, and *D. Jendrossek* (*Figure 10*,**A**).[89] The conclusion of this investigation is: a receptor in our skin responsible for sensing cold is only fully active when PHBylated!

Whether this is also true when TRPM8 exhibits other physiological and pathological activities[86,91] remains to be seen – if it is looked for at all!

It has been suggested that there may be a correlation between the temperature dependent activity of PHB-containing channels and the conformational mobility of the PHB chains.[24,80] If we take the rotational barriers around the single bonds (labelled in *Table 2*) of butane, methyl acetate, *N*-methyl acetamide, and 2-butene as simple reference values, we realize that rotation around the functional groups of PHB at room temperature is *much* faster than that around the amide groups of peptides. Furthermore, we notice that the glass temperature of PHB (*ca.* 5 °C) is in the temperature range of sensing by the TRPM8 protein channel. The constant temperature-dependent movement of PHB chains could influence and change channel activity by interactions with certain amino-acid side chains of the protein. The flexibility and conformational energy of PHB may cause the large enthalpy and entropy changes accompanying the gating processes.[85,89]

The conformational mobility of PHB chains may also be responsible for the fact that post-translational PHBylation is not detected in cryo-electron microscopy (cryo-EM) analysis (*Figure 10*,**B**).[92,93] The flexible PHB chains are present in different conformations, disordered, a mess compared to the orderly crystallites (*Figure 8*) and the comparatively rigid peptide structures. When the sample undergoes shock freezing in water below −150 °C at a rate of over 10,000 K/s,[95] the PHB chains are fixed in their various conformations and not detected by cryo-EM because disorder in the substrate leads to electron density that is smeared out and hence uninterpretable. The same is true for other PTMs, especially for lipidations where the even more mobile fatty-acid chain conformations (*Table 2*) are frozen and likewise lost in the image analysis. Nevertheless, in the papers describing the TRPM8 cryo-EM structure published 2017 and 2019,[92,93] the detailed biochemical structural analysis (published 2013) of TRPM8[89] is not even mentioned or referred to.

Another reason why PHB is not detected in standard protein structural analyses is the chemical instability of PHB: the ester groups are readily cleaved by hydrolysis under acidic *and* under basic conditions, by nitrogen nucleophiles,[96] and by *Lewis*-acid catalysis, and there is pyrolysis above 100 °C. When looking at the typical sample preparation conditions for protein single-crystal X-ray structure determination it is obvious that no care is taken for the sensitive PHB chain: supersaturated solutions are prepared by
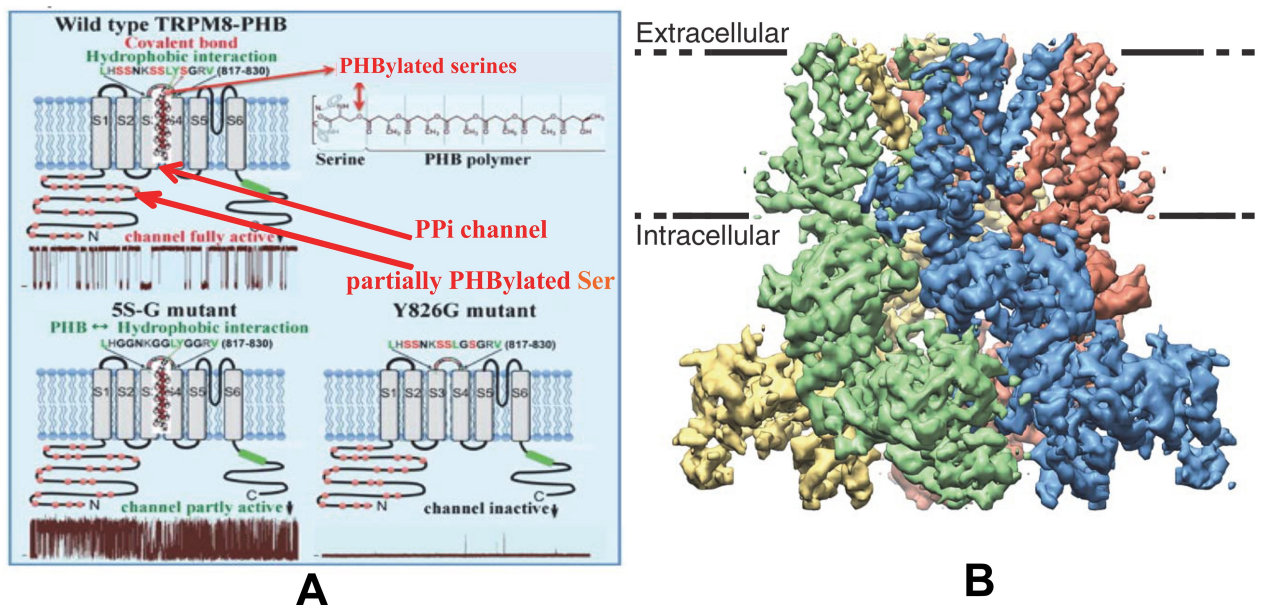
Figure 10. A: Keywords about the biochemical TRPM8 investigation.[89] HEK293 cells, rat DRG neurons, anti-PHB F(ab′)$_2$ antibody, PHB depolymerase PhaZ7-expressing cells, electrophysiological characterization, Ca^{2+} fluorescence imaging, site-directed mutagenesis, post-translational protein modification by PHB (PHBylation), LC/MS, MALDI-MS, MS/MS, whole-cell patch clamp technique, cold, menthol and icilin activation, temperature dependence. This figure is the slightly modified Graphical Abstract from *E. Zakharian*'s article in [89]. Courtesy *E. Zakharian*, 2013, *Cell Reports*.[89] **B**: Cryo-electron-microscopy structure of TRPM8 (4.1 Å resolution)[92] This picture is part of *Figure 1* in ref. [92], copyright 2017, *Science*, granted by The American Association for the Advancement of Science (order number 5493130841886), *cf. Figure 1* in ref. [93].

addition of salts and polymers, and temperature and pH are manipulated.[97]

Finally, and perhaps most importantly, many proteins for structural analysis are synthesized by recombinant protein expression technologies in hosts lacking the relevant PTM machinery!

The results described above leave no doubt about the fact that PHBs of various chain lengths (from 2 to >1000 HB units) play fundamental roles in the chemistry of life. Clearly, methods of detecting PHB are available, and thus numerous hitherto unknown physiological activities of PHBs wait to be discovered and exploited for the benefit of medical health! And yet, statements made approximately 10, 20, or 30 years ago still hold:

- 'PHAs: a Fifth Class of Physiologically Important Organic Biopolymers?'.[98]
- 'PHB merits being the central activity of scores of laboratories and being on every undergraduate syllabus'….'and yet this opus has gone largely unnoticed'. (*Figure 3*, Special issue of *Cell. Mol. Biol.*, DOI 10.1170/T673).
- 'In conclusion, oligo-PHB and cPHB are molecules of essential and fundamental importance in living cells'.[10]

- 'this natural polymer that has been ignored in biochemistry textbooks for a long time'.[99]
- 'cPHB therefore is one of the regulators of metabolic activity. These views place cPHB at the very origins of life, and point to significant roles for cPHB in normal and abnormal cell biology'.[24]
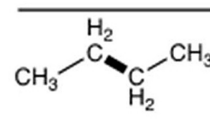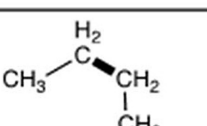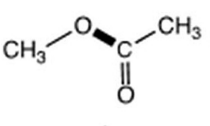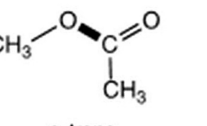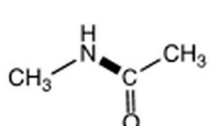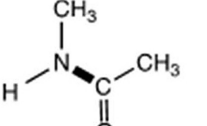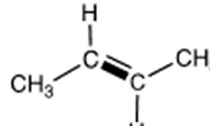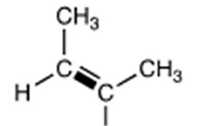
PHB appears and disappears everywhere in the kingdoms of biology – we could call it a 'fleeting' species, so far a curiosity for most scientists.

5. Is PHB a Component of Primordial Chemistry or Has It Been Introduced Late in the Origin of Life? Compartment Formation

Research addressing the subject *origin of life* has increased exponentially in recent decades. For a small, personal collection of articles and books see references.[100–114]3 The contributions to this topic are diverse and cover a wide range of aspects from

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3 Besides reference [103], also go to 'Articles in the Synlett Cluster on Prebiotic Organic Chemistry and Chemical pre-Biology'.

Table 2. Conformational Mobility of the chains in fatty acids, PHB and peptides by comparison with the simple analogs butane, methyl-acetate, *N*-methyl acetamide and 2-butene. Rotational barriers as well as associated rate constants *k* and lifetimes *t* at 298.15 K as calculated with CCSD(T), extrapolated to the complete basis set limit with the cc-pVDZ and cc-pVTZ basis sets. All calculations were done with Turbomole 7.4.1.

more stable	less stable	energy difference	rotational barrier	rotations/sec at 298 K	*cf.* Biopolymer
		Kcal/mol			
ap (antiperiplanar)	*sc* (synclinal)	0.9	3.3	2.3×10^{10}	fatty acid chain
s-cis	*s-trans*	7.5	13.3	1.0×10^{3}	PHB (Tg 5°C)
s-cis	*s-trans*	2.7	21.0	2.3×10^{-3}	peptides, proteins
E	*Z*	1.0	61.9	2.5×10^{-33}	unsaturated fatty acid

Hitherto unpublished results by T. Weymuth and M. Reiher, Laboratory of Physical Chemistry, ETH Zürich

molecules entering the earth through meteorites, to the primordial soup, to prebiotic *Darwinian* evolution, all the way to biomolecular homochirality.[113,114]

In an insight-full statement by *Albert Eschenmoser* the essential point to be drawn is: 'the origin of life cannot be 'discovered', it has to be 're-invented''.

Concerning the role of PHB in this scenario, *Norris, Reusch et al.*[115] have presented a hypothesis about protocells, and *Reusch* has repeatedly pointed to the possible involvement of PHB in a world before other biopolymers.[24][116] Whatever possibilities the many proposals about the origin of life consider,[100–112] at some point there must be *compartmentalization*.[106–112,117]

Based on our experience with the structure and properties of OHBs (*Figure 8*) we can hypothesize, or

better speculate, that under the reductive conditions of fatty acid formation from acetic acid on a mineral surface[100,118] there could have been a bifurcation at the stage of OHB or PHB formation, providing the components of micelles and of vesicles (compartments) with PHB ion channels (*Figure 11*, **A**).

In view of today's cell membranes there are three intriguing questions to be asked: *1*) Why do the fatty acids in cellular bilayers universally have chain length of *ca.* 18 C-atoms, which happens to be the maximum chain length for an unfolded alkane in the gas phase?[119–122] *2*) Is it correct to assume that only monodisperse OHBs form well-ordered ion pores and Ca-PPi-PHB channels through bilayers? (*Figure 11*, **B**, *cf. Figure 8*). If so, pore formation would separate the enantiomeric all (*R*)- and all (*S*)-OHBs from their many
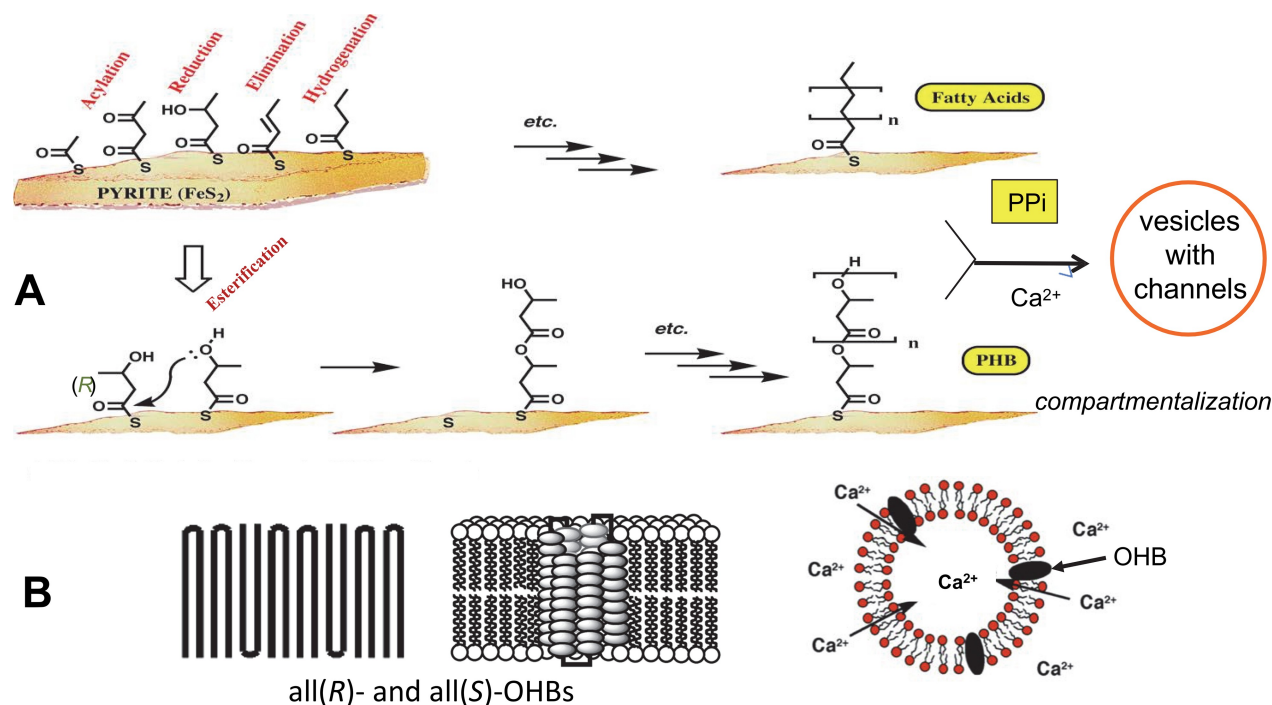
Figure 11. A: Possible parallel formation of fatty acids and of OHBs under prebiotic conditions. In the presence of PPi and Ca^{2+} ions this could lead to vesicles with ion pores and channels (*cf. Figure 8*,**D** and *8*,**E**) and with the Ca-PPi-PHB channel (*cf. Figure 9*). **B**: It is assumed that only monodisperse OHBs, consisting of all(*R*)- or all(*S*)-HB can aggregate to form the well-ordered PHB arrangements in a channel or pore.

polydisperse diastereoisomers. *3*) The most difficult question: at which point, and by which mechanism 'was the decision made' that the absolute configuration of β-hydroxy-butanoates in modern cells would be (*R*) and not (*S*)?[113,114] Question (*2*) could be answered by specific synthesis of polydisperse HB oligomers and comparison with the monodisperse OHBs we have studied (*Figure 8*). Questions (*1*) and (*3*) 'are for' physical chemists and theoreticians, respectively.

The notion that compartments, comprised of fatty acids, may have co-existed in an environment with PHB, PPi, and various ions such as Na$^+$, K$^+$, and Ca^{2+} suggests that *compartmentalization* may have occurred, for instance of divalent *vs.* monovalent ions (cation discrimination), at an early stage of prebiotic chemistry. These biopolymers would, thus, not have been introduced sometime later to a world of nucleic acids and peptides, but *vice versa* might have helped in the development of other biomolecules by providing an early system for compartmentalization and complexation. This would be compatible with the fact that PHB is ubiquitous in living organisms and that it is involved in fundamentally important processes such as carbon and energy storage, various ion pumps and

ion channels, genetic competence and DNA transformation, post-translational protein modification, *etc.*

The word *compartmentalization* triggers the synthetic organic chemist to make a connection to a recent development in his field: the use of 'interface-rich aqueous systems (IRAS) that promote and guide chemical reactions'.[108] This includes micellar catalysis,[123–125] phase transfer catalysis',[126–128] use of emulsion droplets,[108,110,111] reactions 'on water',[129] in water and in deep eutectic solvents.[130–134] Two examples published by *V. Capriati* and his group are presented in *Figure 12*.[131] A finely divided suspension of an organic electrophile in water is treated with three equivalents of an ether or hydrocarbon solution of a polar organometallic reagent, and *ca.* one third of this nucleophile succeeds in meeting electrophile (through droplet-droplet fusion?), rather than being hydrolyzed.

It is hoped that this article will generate interest and enthusiasm for PHB among those teaching biology and medicinal chemistry and among their students in the next generation of natural scientists. This intriguing biopolymer is likely involved in many hitherto unknown physiological processes. A greater understanding of its biological roles could eventually

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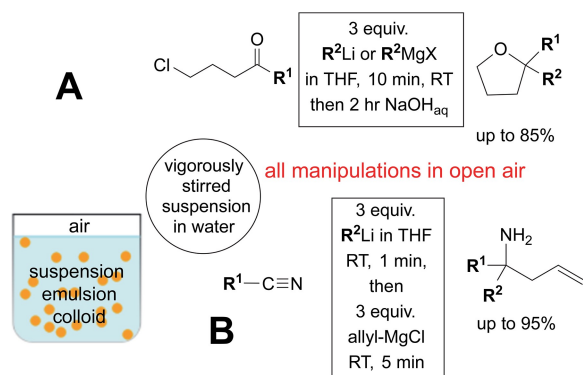
Figure 12. Compartmentalization in organic synthesis. A solution (3 equiv.) of a Li compound or of a *Grignard* reagent in THF or in an ethereal hydrocarbon is added to a vigorously stirred suspension of an electrophilic organic starting material in water. All operations are done in open air and the reactions take just a couple of minutes each! **A**: Conversion of γ-chloroketones to 2,2-disubstituted tetrahydro-furans. **B**: Conversion of nitriles to 1,1-disubstituted homoallyl amines. See references cited in [130–134].

lead to a complete shift in paradigms – a late reward for the work of *Rosetta Reusch*!

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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