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An Introduction

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Preface

This book sums up ten years' work, carried out mostly in the author's laboratory, reporting data and observations related to low temperature biochemistry.

Such a project offers the opportunity to collate published data and observations, not as a simple compilation, but as a comprehensive study and potential tool for future investigators.

The author is aware of the fact that the methodology can largely be improved upon as can the devices built or adapted to such studies. While the References are by no means comprehensive they should lead the reader to more extensive reviews which contain a fuller list of references to the original literature. Thus, the book presents both the defects and, we hope, the advantages of a personal experience at its very early stage of development, and should be considered merely as an introduction to an emerging methodology.

This book has grown out of a course at the Illinois University during the academic year 1974–5 and owes very much to the stimulating and friendly climate created there by Professor I. C. Gunsalus. The kind hospitality of Dr. E. Zuckerkandl at Saint Guilhem Le Desert provided a wonderful opportunity to try to make a synthesis of the experimental work carried out over years at the Institut de Biologie Physico-Chimique in Paris, a wonderful place to work among distinguished scientists and friends such as Alberte and Bernard Pullman, Sabine and René Wurmser, Marianne Manago, Mike Michelson, Pierre Joliot.

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Pierre Douzou
February 1977

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The author trusts that due acknowledgements have been made in all cases and apologizes for any omission that may have been made inadvertently.

P.D.

To

CLAUDE BALNY, GASTON HUI BON HOA,

PASCALE DEBEY, FRANCK TRAVERS, PATRICK MAUREL

whose efforts made this monograph possible

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Introduction

The essence of truth is superior to the terminology of "How"? or "Why"?
Hakim Sanaī XIth century

Why?

One of the most important aspects of biochemical processes is their dynamics and the measure of their overall rate is in fact the measure of the rate of the slowest of the series of successive reaction steps involving structural and catalytic transformations. All the steps should be known to explain any reaction mechanism but most of them are so fast that they go to completion in times going from the millisecond to a fraction of a nanosecond and necessitate rapid kinetic techniques. The fastest biochemical reactions are now accessible to experimental measurements since time resolutions of the order of a nanosecond are attainable, but many of the present rapid techniques give nothing more than a characteristic "relaxation time" with no indication about what this time refers to, since measurements involve optical spectroscopy rather than the more sophisticated techniques essential to determine the structures but which are not adapted to recordings in very short time ranges.

Thus new procedures are needed which would permit all the required structural information about any biochemical system to be obtained as a function of time. Several years ago, we decided to explore in this laboratory the possibility of slowing down reactions by temperature-effect, since almost all biological processes are temperature-dependent and, were it not for the crystallization of water, it is probable that most biochemical systems could be reduced in temperature sufficiently to reduce their reaction rates enormously, thus to improve their analysis, and even to induce complete suspension of their activity.

Moreover, since most biochemical processes consist of several successive steps, the effect of temperature on their overall rates will be the sum of its separate effects on these steps according to their temperature coefficients and activation energies, hence lowering the temperature should consequently lead to a thermal resolution of the individual steps.

In the case of enzyme-catalyzed reactions which consist of at least three consecutive steps

$$E + S \rightleftharpoons E-S \rightleftharpoons E-P \longrightarrow E + P$$

involving at least 6 thermodynamic parameters for each step (namely heat, free energy and entropy of both activation and of the process itself), a very small number of them can be determined and their relationships are unknown because very few separate velocity constants have been obtained and the measurements have been carried out only at one selected temperature. The low temperature procedure should therefore shed a new light and enhance our knowledge of forward and backward reaction pathways in biochemical reactions.

In the field of analytical biochemistry, the eventual adaptation of some of the procedures for the fractionation and concentration of lipid and protein components of biological tissues might open new interesting ways: column chromatography techniques, used at subzero temperatures, should permit the stabilization of some thermodynamically labile enzymes, the concentration and isolation enzyme-substrate complexes, and finally the improvement in resolution.

The introduction of a suitable low temperature procedure could even revive the interest and increase the usefulness of the old methods of salt and organic solvent fractionation which appear to have fallen into disuse, but offer certain advantages when compared to the newer procedures and which can use the solvents employed, as we will see later, to cool our media. Because of the lower density of these mixed solvents, compared to concentrated salt solutions, shorter times or lower speeds of centrifugation would be sufficient to collect the precipitated components and here again new resolution possibilities might be found.

The fact that exposure to organic solvents at room as well as at subzero temperatures can denature proteins is presumably responsible for the neglect of organic solvent fractionation as a technique, since the methods used in analytical biochemistry ought to isolate and concentrate valuable components as nearly as possible in their natural state. Any procedure which retains at low temperature the properties of water as a solvent of proteins and its fluidity would certainly induce a new surge of interest of older fractionation techniques.

The low temperature procedure could be applied to the elucidation of the intermediate steps in metabolic processes. On cell-free extracts, metabolic intermediates can be accumulated by use of specific inhibitors or by inactivation of specific enzymes. Substrates normally in too small a concentration to be detected will accumulate and will be isolated by lowering the temperature enough to interrupt the sequence of reactions at a given step.

Such a method could replace the use of specific "poisons" with greater flexibility and total reversibility.

Another potential application of cryobiochemistry could be cryobiology—a discipline concerned with the singular and yet largely unexplained events associated with life and death at low temperatures, primarily below freezing, of living systems. Cryobiology is almost entirely dominated by its applications, consisting mainly of organ banking; the remarkable developments which have occurred in recent years in surgical "grafting" techniques require methods for prolonging the viability of the organs *in vitro*, awaiting transplant, below zero degrees.

Thus cryobiology is faced with the necessity of finding methods of keeping cells dormant but potentially alive by cooling or freezing at subzero temperatures. This is a challenge since it is known that water does crystallize to ice with a multitude of consequences which are fatal to most of living systems; ice-crystals produce histological injuries both by mechanical effect and dehydration as water is removed to form ice.

Dehydration itself is known to cause the following changes: increase in electrolyte concentration with a resulting increase of ionic strength of the suspending medium and precipitation of proteins from solution, changes in pH, concentration of certain solutes to toxic levels (urea, gases etc.) sufficient removal of water to bring structures into actual physical contact and leading to abnormal cross-linking, removal of structurally essential water, and so on.

Some of these consequences of freezing seem to be avoided by macro addition of "cryoprotective" agents (glycols, polyols, dimethylsulfoxide) but current level of knowledge in cryobiology is still insufficient to explain manifestations of cryoinjury and cryoprotection at any level of biological complexity. Up to now, the approach of such problems have been too empirical and basic information is essential to understand the causes and consequences of subjecting cells to subzero temperatures.

It is within cells, cell-free extracts and biochemical enzyme-dependent relationships that cryoinjury and cryoprotection should be studied. The chief limitations of such observations are due to freezing, so cooling above freezing might open the way to such studies.

Joint *in situ* and *in vitro* experimentation of enzyme-catalyzed reactions and of multi-enzyme processes leading to biological functions could be performed before, during and after cooling, and provide background informations still lacking in cryobiology.

Finally, cosolvents and temperature variations could be used as tools to investigate the macromolecular basis of catalysis and the kinds of rearrangements that proteins undergo both under the influence of substrates and of effectors. In some cases is should be possible to observe such rearrangements by X-ray analysis of enzyme-substrate intermediates stabilized in the

crystalline state at subzero temperatures. In other cases these rearrangements could be detected in solution through the reversible perturbations determined by cosolvents as opposed to substrates and effectors, and recorded by various methods.

Thus, there are a number of obvious advantages in carrying out low temperature biochemistry but the question is how to succeed in such an enterprise, a problem which will now be examined and then analyzed at length in chapters 2 and 3.

How?

The requirement for performing low temperature biochemistry is the prevention of water from freezing by the macro addition of miscible organic solvents without altering the capacity of water as a solvent of biomolecules in their ionic environment. This is *a priori* a difficult if not impossible task since water is unique in its structural and physico-chemical properties which determine the structural integrity of biological components and of their systems.

Highly polar water molecules give rise to electrostatic forces of high magnitude and these properties will be affected by any addition of "non-biological" components even when they are highly polar in themselves and give mixed homogeneous solutions retaining many of the properties of pure water. The possibility of special effects on structural and catalytic integrity of proteins due to the addition of miscible organic solvents to water as "antifreeze" has caused caution among biochemists in the application of low temperature procedures and therefore the advancement of cryobiochemistry.

As stated more than a century ago by Claude Bernard, the properties of the internal environment of living systems must be kept constant within very narrow limits, for biomolecules are exceedingly sensitive to small changes in physico-chemical properties of their immediate surroundings. The presence of organic solvents depressing the freezing point of solutions of biomolecules indeed alters the properties of the original purely aqueous medium, these alterations being proportional to the concentration of dissolved solvents.

Thus, by the addition of a solvent on the one hand and lowering the temperature on the other, we will alter the delicate balance maintaining the physico-chemical properties of biological systems at constant values and henceforth their structural and catalytic integrity.

In fact, there are some amendments to this rule since it is found in Nature that some cold-blooded species perform the synthesis of organic substances to "supercool" and then to prevent a substantial portion of their body water from freezing.

Resistance to cold in Nature

Resistance to cold in Nature is a fascinating and encouraging example of the eventual possibility of preventing water from freezing by the presence of organic substances without altering its capacity as a reactive component and as a solvent of biological systems.

It is known that insects hibernating in temperate or cold regions can avoid freezing injury by "supercooling" after accumulation of small molecular weight compounds (polyols and sugars) which depress their body fluid freezing point. Most poïkilotherm organisms (micro-organisms, plants and animals) do not freeze during the winter season, in some cases at temperatures as low as $-35°C$.

In many insects, polyol formation has been found to occur only during diapause, that is in the larval or pupal stage, but in some adult insects such as the carpenter ant or an Alaskan beetle, seasonal fluctuations of polyol content have been observed which mirrored fluctuations in ambient temperature.

The polyol is glycerol which is formed from glycogen. Sugars such as sorbitol and trehalose are also found in some species. The concentration of glycerol, expressed as a percentage of fresh body weight, can be as high as 25% in the larvae of *Bracon cephi* (hymenoptera), or as low as 1.1% in the eggs of *Bombyx mori* (lepidoptera) where 2.2% sorbitol was also detected.

In high concentrations, glycerol lowers the freezing point of the haemolymph to about $-15°C$ and can determine a supercooling as low as $-47°C$.

In the adult beetle *Pterostichus brevicornis* (Alaskan), direct correlation between haemolymph glycerol content and supercooling and freezing points have been found in both naturally and laboratory acclimatized forms. Mean glycerol level was less than 1 mg% during summer and increased to 22 mg% during winter, the supercooling points being respectively $-4.2°C$ and $-11.5°C$, the freezing points $-0.6°C$ and $-5°C$.

As we mentioned above, glycerol seems to originate from carbohydrate precursors, as indicated for instance by high fructose diphosphatase activity levels in winter and glycerol's rapid conversion to glycogen when winter acclimatized species are taken into the laboratory. On the other hand, the fat content as a percentage of dry body weight declines from about 30% in the fall to about 20% for the winter and spring in the case of Alaskan beetles.

Some insects seem to use sugars to prevent cold injury; diapausing prepupae of the Japanese fly *T. Populi* accumulates sugars at the beginning of the fall; the total sugar levels average 5 to 7% of fresh body weight, of which more than 90% is trehalose. A clear correlation between sugar content and frost resistance is observed in these insects during the period from August to

June. During the cold season, none of the prepupae are killed in liquid nitrogen provided they were previously frozen at $-30^{\circ}C$.

Finally, we can say that a slight increase in glycerol or sugar content is always followed by a remarkable increase in frost resistance capacity of most of insect species, and the mechanism of the protective action of glycerol in these cases may well differ from the mechanism operating in the protection of mammalian cells and tissues found in cryobiology, protection which confirms the possibility of the use of organic additives for low temperature biochemistry.

After reconversion of glycerol or sugars to glycogen, insect larvae are able to complete metamorphosis, demonstrating that both organization and function are unaffected by the presence of such solutes.

Prevention of freezing injury: cryobiology

Apart from the abilities of poïkilothermic organisms to avoid freezing and its fatal consequences, it was discovered independently in 1949 that some organic substances behave as protective agents against freezing injury to certain mammalian cells.

Glycerol was the first of these substances to be used successfully and is still the leading cryoprotective agent, presumably owing its comforting virtue of being a normal intermediary product of lipid metabolism, both in mammals and insects.

On the other hand, some "non-biological" substances such as dimethylsulfoxide, ethylene glycol and propylene glycol can be used as cryoprotectives. Nevertheless, in spite of the considerable amount of literature on such additives, the basis of their action still remains largely obscure.

The requirements for use are their nontoxicity, their ability to penetrate the cell membrane freely and their capacity to dissolve electrolytes. All contain hydrogen-bonding groups (OH, NH, $=0$) and bind strongly with water, preventing it from freezing at $0^{\circ}C$. Their protective action can be understood in the cooled state since it is known that water crystallizes to ice with a multitude of consequences. Let us recall that the production of ice crystals determines histological injuries and also dehydration as water is removed to form ice; dehydration causes several changes including a resulting increase in the ionic strength of the suspending medium hence precipitation of proteins from solutions, changes in pH, abnormal concentration of solutes such as dissolved gases, urea, etc. to toxic levels; finally, sufficient removal of water could bring structures into actual physical contact, leading for instance to abnormal cross-linking.

It is difficult to offer an explanation of how such effects could be avoided during freezing of media containing cryoprotective additives, but here again

we have to stress that the presence of high concentrations of organic and eventually "non-biological" solvents does not alter irreversibly the functioning of most of biological systems.

Physical-chemical basis of low temperature biochemistry

Besides the action of organic solvents in cryobiology and their innocuity towards biological systems, it must be remembered that many soluble (purified) enzymes are currently dissolved in water–glycerol mixtures and stored at low temperature were they are found to be "cryoresistant", and also that certain enzymes which consist of subunits are protected against irreversible inactivation during freezing-thawing in the presence of large amounts of polyols. However, storage conditions can be quite different from conditions insuring optimal activity.

Before the beginning of our own work, very few attempts to carry out enzyme catalyzed reactions in mixed solvents at subzero temperatures were reported in the literature (3), (4), (5). Among them the author would especially mention the work of Simon Freed who injected solutions of enzymes in water cooled at 0°C as a fine spray into several cooled alcohol–water mixtures, obtained the progressive dissolution of the small "icebergs", showed that the hydration of the enzymes persisted at low temperature in predominantly alcoholic solvents, and demonstrated the occurrence of reactions in such conditions (6). Freed understood the potentialities of the procedure as well as the absolute necessity to learn more about its basis and was a pioneer in the field of a low temperature biochemistry.

Nevertheless, most purified enzymes and cell-free biological systems might be sensitive to the macro-addition of organic solvents. Such an addition not only decreases the freezing point of aqueous systems but indeed alters physical properties such their as molecular volume, viscosity, dielectric constant, which in turn influence the behavior of structural and catalytic biomolecules, their reaction rates and eventually their mechanisms.

The broad variations in solvent properties, first on addition of "antifreeze" solvents and then on cooling of the mixtures, are at least hypothetically accessible and the documentation of their numerical values is necessary both to predict and explain or even to correct such variations so that the alteration of the structure and function of biomolecules could be minimized.

The physical chemistry of mixed solvents in the range of normal and subzero temperatures has been carried out mainly in this laboratory and will be treated at length in chapter 2.

For the moment, since we are dealing with the "how" of an eventual low temperature biochemistry, we can try to get a bird's eye view of this essential problem.