Cryoenzymology: The Use of Fluid Solvent Mixtures at Subzero Temperatures for the Study of Biochemical Reactions

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Why low temperatures?
A reaction with an activation energy of 60kJ/mol will be decreased in rate by 100-fold if the temperature is decreased from 20 to −30°C. Depending on the rate of the reaction at normal temperatures, a decrease of this type can have a number of valuable effects.

(i) Reactions normally occurring in a few seconds or minutes may be sufficiently decreased in rate to permit reaction intermediates to be purified by column chromatography or even to allow the determination of their structure by X-ray-diffraction techniques.

(ii) Reactions normally occurring in the millisecond range and requiring specialized rapid-reaction techniques for their study can be reduced to the time scale of seconds. This is of particular interest in the case of reactions involving small changes or requiring the use of techniques such as dual-wavelength spectrophotometry.

(iii) Reactions occurring in the microsecond range and only accessible to relaxation techniques of limited applicability (such as temperature jump) can be studied by rapid-mixing temperatures at subzero temperatures provided suitable apparatus can be developed. In this case, low temperatures provide a way of circumventing the technical barrier caused by the difficulty of mixing two solutions in less than a millisecond.

In all cases, decreasing the time over which the reaction occurs will increase the precision of experimental recordings and may allow one measurement to take the place of repetitive ones or the scanning of a spectrum to take the place of measurements at a single wavelength. A further interest of low temperatures is that different steps of a reaction sequence may be affected to different extents, allowing the possibility of observing intermediates that do not occur in measurable quantities at normal temperatures.

There is thus great potential interest in the use of low temperatures for the study of biochemical reactions (sometimes called cryoenzymology). However, useful decreases in rate frequently require the use of temperatures below −20°C. Even in the rare cases when a light-initiated experiment can be devised, the use of solid media poses its own problems and it may be preferable to avoid freezing. For reactions started by mixing a liquid medium is essential. Low-temperature biochemistry is therefore mainly concerned with the use of fluid solvent mixtures.

Development of the approach

Such an apparently obvious way of studying rapid biochemical reactions has been little used until recently. A number of pioneering attempts are cited by Douzou (1974), but the approach has been relatively neglected until the work of P. Douzou and his collaborators in Paris and Montpellier. Douzou's rationale was that biochemistry at subzero temperatures in fluid solvent mixtures was only possible if a range of physicochemical data on possible media was available. Largely as the result of the work of the French group, such information is now available (Douzou et al., 1976; Douzou, 1977), and it has removed much of the guesswork from planning low-temperature experiments. Douzou and his colleagues have also developed some specialized apparatus for this type of work. Building on these foundations the group, and others working in collaboration with them, have successfully applied the low-temperature approach to a variety of systems, and the feasibility of such studies has been confirmed by other groups working independently. Subjects investigated include d-amino acid oxidase (Shiga et al., 1967), horseradish peroxidase, etc.
Table 1. Temperatures required to obtain various decreases in reaction rate compared with that at 20°C

<table>
<thead>
<tr>
<th>Activation energy [kJ/mol (kcal/mol)]</th>
<th>Temperature (°C)</th>
<th>Decrease in rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 (9.5)</td>
<td>10²</td>
<td>-44</td>
</tr>
<tr>
<td>100 (23.8)</td>
<td>10⁴</td>
<td>-85</td>
</tr>
</tbody>
</table>

peroxidase (Douzou et al., 1970), bacterial luciferase (Hastings et al., 1975) and carboxypeptidase A (Makinen et al., 1976), as well as the areas discussed below.

No attempt will be made here to discuss all the results obtained at low temperatures, or give a detailed theoretical background. These have been done in a book by Douzou (1977) as well as in several reviews (Douzou, 1974; Fink, 1976a; Makinen & Fink, 1977). Instead, after a brief outline of the principles of the low-temperature approach and some of the practical problems, a few examples of recent work will be examined to show the type of insight that the use of low temperatures can bring to biochemical problems.

Principles and problems

Effect of temperature on chemical reactions. Experimentally, the dependence of the reaction rate constant, \( k \), on the temperature is often found to obey the simple Arrhenius expression:

\[
k = A \exp(-E_a/RT)
\]

where \( A \) is a constant for the reaction, \( R \) the gas constant and \( T \) the absolute temperature. Reactions obeying this relationship give linear Arrhenius plots of logarithm of the rate constant against \( T^{-1} \); the gradient of this curve is \(-E_a/R\) and the value of \( E_a \) is the 'apparent Arrhenius activation energy'. For complex reactions this has no ready interpretation in terms of transition-state-complex formation, but it provides a convenient way of characterizing the temperature-dependence of a reaction. Most biochemical reactions have Arrhenius activation energies of between 40 and 100 kJ/mol. Table 1 shows the effect of temperature on the decrease in rate for these two values.

Choice of solvent. Replacement of normal aqueous media by those containing high concentrations of organic solvents frequently has an inhibitory effect on biochemical reactions.

The least damaging solvents appear to be polyols or dimethylsulphoxide, which may inhibit merely by decreasing the water concentration. Solutions containing glycerol are too viscous to be of practical use in most low-temperature applications and ethanediol (ethylene glycol) seems to be the solvent of choice for many biochemical experiments. A commonly used medium is a 1:1 (v/v) mixture with water, which is fluid down to \(-40°C\), where it has a viscosity of 125 cP.

Fink (1976a) has used dimethylsulphoxide in studies on a variety of enzymes. Many of his experiments are done with 65% (v/v) dimethylsulphoxide at temperatures down to \(-90°C\). Dimethylsulphoxide seems to be a valuable solvent particularly at these very low temperatures where polyols cannot be used.

For rapid-mixing experiments the high viscosity associated with polyols may be a severe limitation and methanol has been used in such situations. The combination of low viscosity and low freezing point would make methanol the ideal solvent, were it not for its specific denaturing effect on many biological systems. Synchronization of solvent addition and cooling may decrease the extent of denaturation, or ternary mixtures containing both methanol and ethanediol (Travers et al., 1975) can be used as a compromise (Makinen et al., 1976). In general it is when using solvents such as methanol that
complicated experimental protocols are necessary and a certain amount of trial and error may be needed to find suitable experimental conditions. In the case of polyols, the biochemical preparation can usually be simply added to the aqueous/organic medium at a temperature just above 0°C. In all cases it is desirable to avoid the addition of aqueous solutions to media at subzero temperatures.

**Physicochemical properties of the media.** An aqueous/organic mixture below 0°C has physicochemical properties that differ from those of an aqueous solution at normal temperatures. These must be considered when planning biochemical experiments. Some of the changed properties can be compensated for, now that a range of data is available, and this is discussed here. Unavoidable effects of solvent and temperature on biological materials are discussed in the next section.

Perhaps the most important factor is the effective protonic activity, denoted by pH*. Values of pH* were originally measured with indicators, starting from the assumption that 10 mM-HCl was completely ionized even in aqueous/organic mixtures (Hui Bon Hoa & Douzou, 1973a). Larroque et al. (1976) described the use of a modified glass electrode for the direct measurement of pH* in cooled aqueous/organic mixtures. Subsequent experiments with biochemical systems have provided empirical confirmation of these pH* values.

Values of pH* depend on solvent and temperature in a way that varies for different buffers, but with the data available, a medium of known pH* under given conditions may be prepared. An example of the effect of solvent and temperature is provided by Tris/HCl buffer; a solution of this at pH8.0 in water at 20°C will be pH*10.5 in 50% (v/v) ethanediol at -40°C (Hui Bon Hoa & Douzou, 1973a). The pK values of groups on the protein may also be affected by temperature and solvent, so adjusting the pH* to a pH that is suitable at normal temperatures may not be sufficient.

The dielectric constant of the medium will affect the strength of electrostatic and hydrophobic interactions, but in practice it does not seem to be especially important. The dielectric constant of a mixed aqueous/organic system increases as the temperature is lowered, and the bulk addition of glycine provides a way of further increasing its value (Travers & Douzou, 1974).

Excessive viscosity causes practical problems in handling the media, makes rapid mixing impossible and will affect the rate of a diffusion-controlled reaction. Viscosity can only be controlled by choice of the organic component of the medium.

**Inevitable effects of solvent and temperature.** For the majority of biochemical systems it has been relatively simple to find a fluid medium in which activity is retained at subzero temperatures. This suggests that many biological macromolecules can adopt an active conformation in a variety of different environments. Even in the case of systems associated with biological membranes the effects of aqueous/organic mixtures at low temperatures seem surprisingly small, whether for microsomal preparations (Debey et al., 1973), mitochondria (Erecińska & Chance, 1972), chloroplasts (Cox, 1975a) or respiratory particles from the bacterium *Escherichia coli* (Pudek & Bragg, 1976). Possibly the arrangement of the hydrophobic membrane interior is little affected by changes in the external solvent.

In the case of biological membranes a special temperature effect must be considered. Membrane lipids undergo changes from a liquid to a crystalline state as the temperature is lowered [for a review see Melchior & Steim (1976)]. These raise the possibility that at subzero temperatures the membrane lipids may be in different states from those at normal growth temperature.

The membranes of organelles such as mitochondria or chloroplasts may be less permeable to the organic component of the medium than to water (Wang & Nobel, 1971; Nobel, 1973), raising the possibility that under some conditions the organelles could be transiently subjected to extremely high osmotic pressures. However, this does not seem to be a problem in practice, at least in the case of chloroplasts and ethanol (Cox, 1975a).

**Apparatus for low-temperature experiments.** Most spectrophotometers and similar apparatus can be easily and cheaply adapted for low-temperature studies. Cold N₂ gas
provides the most flexible cooling system (Maurel et al., 1974), but for many purposes a circulating liquid cooled in a cryostatic bath is perfectly adequate.

More specialized apparatus has also been developed to take full advantage of the potentialities of the low-temperature technique. These include a stopped-flow spectrophotometer (Hui Bon Hoa & Douzou, 1973b), which will be discussed below in the context of the results obtained using it in studies on cytochrome P-450, and a 'slow' temperature-jump device with a time constant of 1s for studying reactions after a sudden change in temperature (of as much as 50°C) and possibly pH* (Hui Bon Hoa & Travers, 1972).

Applications of the subzero-temperature approach to biochemical problems

Serine proteinases: low-temperature column chromatography and protein crystallography. These are a well studied class of enzymes involved naturally in protein breakdown. Different members of the class such as elastase and chymotrypsin have different specificities, but a similar catalytic mechanism involving a reactive serine residue at the active site, which is generally agreed to be involved in the formation of a covalent acyl-enzyme intermediate. [For a recent review see Kraut (1977).]

Low-temperature studies have made a number of interesting contributions to the investigation of these enzymes. Fink and his collaborators have made a series of studies of chymotrypsin at temperatures down to -90°C with media containing 65% (v/v) dimethylsulphoxide. At low temperatures the acyl-enzyme could be formed, but the rate of deacylation was so slow that the intermediate could be separated from excess substrate by gel filtration on Sephadex LH-20 at -40°C (Fink 1973a,b). This provided direct evidence for the participation of an acyl-enzyme in the catalytic pathway with a specific substrate.

More recently Fink has used even lower temperatures to study possible intermediate stages in the formation of the acyl-enzyme. A tetrahedral intermediate (with a covalent bond between the substrate carbonyl carbon atom and the oxygen atom of the active-site serine) (Scheme 1) had been suggested by analogy with non-enzymic reactions, and with rapid-reaction techniques spectrophotometric evidence has been obtained for an additional intermediate before the acyl-enzyme in the case of chromophoric substrates (see references cited by Kraut, 1977). By using first the protein fluorescence emission (Fink & Wildi, 1974) and then the absorbance changes during the reaction with N-acetyl-L-phenylalanine p-nitroanilide (Fink, 1976b) it was possible to detect a number of intermediates before the formation of the acyl-enzyme under conditions where deacylation was negligible.

From the spectrophotometric studies, Fink (1976b) proposes four reactions before acylation. Formation of the Michaelis enzyme-substrate complex is very fast, even at -90°C, too fast to study even with the double-syringe device used to fill the spectrophotometer cuvette with a rapidly mixed solution. Changes attributed to two conformational changes follow, with kinetics that can be easily measured at -50°C, and then a third pH-dependent reaction appears to involve the histidine residue adjacent to the active site serine. (This histidine residue, together with the adjacent 'buried' carboxylate group of an aspartate residue, is involved in the charge relay system, which can be regarded as providing a proton-binding site during the formation of the acyl-enzyme.) A fourth reaction

Scheme 1. Formation of the acyl-enzyme during catalytic reaction of serine proteinases

The postulated tetrahedral intermediate is shown within square brackets.

O
R–C + HO–enzyme \rightarrow [O^–] R–C–O–enzyme \rightarrow [X^-] X–H^+

R-C-O-enzyme + XH

1978
was interpreted as formation of the acyl-enzyme; deacylation was too slow to measure under these conditions. It is interesting that no step with the properties corresponding to those expected for the formation of the tetrahedral intermediate could be detected.

The extreme stability of the acyl-enzyme of serine proteinases has been exploited in another way. Petsko (1975) has developed a range of techniques for replacing the mother liquor of protein crystals by aqueous/organic mixtures that are still fluid at low temperatures. It is known that enzyme crystals may be catalytically active at normal temperatures, and Fink & Ahmed (1976) were able to obtain up to 80% formation of the acyl-enzyme intermediate with crystals of chymotrypsin and trypsin suspended in a medium containing 52% dimethylsulphoxide at temperatures below -50°C. In the case of another serine proteinase, elastase, the acyl intermediate could be obtained in a medium containing 70% methanol at -55°C. The acyl-enzyme was formed within 1 day, but was stable for more than 1 week, allowing the three-dimensional structure to be determined by X-ray-diffraction methods (Alber et al., 1976). A difference Fourier synthesis between the native and acyl-enzyme was in agreement with the acyl portion of the substrate being close to the β-oxygen atom of the serine residue at the active site. It was not possible to remove the bound substrate by washing with substrate-free medium, providing evidence for covalent attachment.

These interesting results illustrate that low temperatures can convert protein crystallography into a technique for studying reaction intermediates in enzymology, an approach with enormous potential.

Cytochrome c oxidase: trapping of intermediates by further lowering of the temperature. Cytochrome c oxidase is the terminal component of the mitochondrial electron-transport chain and is involved in the reduction of O₂, a reaction that has been extensively studied. The molecule contains two haem groups, a and a₃, and two copper atoms (for a review see Nicholls & Chance, 1973). Chance and his colleagues (Chance et al., 1975a,b,c) have been able to obtain evidence for several new intermediates by studying the reaction at subzero temperatures. The essence of the Chance procedure is that intermediates formed at one subzero temperature are stabilized by rapidly lowering the temperature further, allowing spectral and other evidence to be obtained at leisure.

In the case of cytochrome c oxidase, Chance was able to take advantage of the fact that the enzyme binds CO competitively with O₂, and that the CO derivative can be decomposed by a flash of light. At normal temperatures the exchange of CO with O₂ is too rapid to allow reactions to be started by a flash except after rapid mixing (Greenwood & Gibson, 1967). However, at -20°C in a fluid medium containing ethanediol the exchange is slow enough for addition of O₂ (by stirring the medium) followed by further cooling to allow a mixture of O₂ and carbonmonoxy-cytochrome c oxidase to be trapped at still lower temperatures, in a medium that is now frozen. The CO derivative was then decomposed by a flash of light and in the presence of suitable O₂:CO ratios the photodissociated enzyme will mostly recombine with O₂. After a flash at -114°C a stable compound 'A' was formed, identified as an oxygenated cytochrome oxidase. The effect of O₂ concentration of the reaction showed that complex-formation was reversible. At this temperature no absorbance changes at 830 nm were observed, suggesting that changes in optically detectable copper were not involved. However, at -96°C the compound A was unstable and was converted into a second compound 'B' in a reaction corresponding to copper oxidation as well as a change attributable to iron oxidation. This compound was stable at temperatures below -60°C. These reactions were interpreted as follows:

\[ \text{Cu}^{+}a_{3}^{2+} + \text{O}_2 \rightarrow \text{Cu}^{+}a_{3}^{2+}\text{O}_2 \]  (compound A)

\[ \text{Cu}^{+}a_{3}^{2+}\text{O}_2 \rightarrow \text{Cu}^{2+}a_{3}^{2+}\text{O}_2^{2-} \]  (or Cu^{2+}a_{3}^{2+}\text{O}_2\text{H}_2)  (compound B)

At suitable temperatures compounds A and B have sufficiently long lifetimes to be trapped by a further lowering of the temperature to that of liquid N₂ (77K) or below, and optical difference spectra were obtained. E.p.r. spectra of compound B trapped under these conditions did not have the signal expected for a structure containing O₂⁻, provid-
ing further evidence that the oxygen in compound B was reduced to the level of the peroxidase. However, the interpretation above must remain tentative until further results have been obtained.

Compound B was stable below $-60^\circ C$; only above this temperature could electron transfer to cytochrome $a$ be observed.

These intermediates have not been observed at normal temperatures even by using rapid-reaction techniques, either because the reactions are too fast or because the intermediates do not occur in detectable concentrations when the relative rates of the various steps are those at normal temperatures. The use of low temperatures has therefore allowed a significant increase in our knowledge of the reaction of cytochrome $c$ oxidase with $O_2$. The precise experimental conditions used by Chance are obviously limited to reactions that can be started by a flash of light, but the general principle of initiating a reaction at a temperature at which it is sufficiently slow to allow intermediates to be trapped by further drop in temperature has a much wider potential application. This type of experiment can be considered as a logical extension of the freeze–quench approach (Bray et al., 1973) with the advantage that slower cooling is possible giving samples suitable for optical as well as e.p.r. measurements.

**Bacterial cytochrome P-450: stopped-flow spectrophotometry at subzero temperatures.** Cytochrome P-450 , is a haem protein involved in the metabolism of camphor by the bacterium *Pseudomonas putida*. It has been the subject of a number of low-temperature studies involving the stabilization of oxygenated derivatives to allow spectra to be recorded (Eisenstein et al., 1977) and the separation of these complexes from redox reagents used in their preparation by gel-filtration at subzero temperatures (Debey et al., 1976; Balny et al., 1976). Low temperatures have also been used to study the kinetics of the reaction of the enzyme with its substrate by stopped-flow spectrophotometry.

Technical problems cause a lower limit of about 1 ms on the resolution of any rapid-mixing apparatus. Mixing efficiency depends on viscosity, but not on temperature, and so the use of low temperatures is obviously a way of increasing the range of reactions that can be studied in this way. The first utilization of these possibilities was in purely chemical studies with reactions in non-aqueous solvents. Allen et al. (1960) built a stopped-flow spectrophotometer that was used to study proton-transfer reactions in diethyl ether/ethanol (4:1, v/v) between $-60$ and $-100^\circ C$ (Caldin et al., 1968).

The design of a stopped-flow apparatus for biochemical studies at subzero temperatures is subject to a number of considerations not usually significant in chemical experiments. The use of aqueous/organic mixtures will mean solvents of high viscosity (particularly if methanol is not suitable) and it is desirable to minimize the amount of solution necessary and maximize the optical pathlength of the measuring cell since biochemical materials are often in short supply. Apparatus designed primarily for chemical experiments such as that of Allen et al. (1960) and more recent designs capable of operating at subzero temperatures (Caldin et al., 1973; Michael et al., 1974) are probably not really convenient for biochemical measurements; large volumes are necessary and no information is given about performance with viscous solvents.

Hui Bon Hoa & Douzou (1973b) designed and built a low-temperature stopped-flow spectrophotometer specially for biochemical studies. This can be used with solvents with a viscosity 50 times that of water at room temperature [ethanediol/water (1:1, v/v) at $-20^\circ C$].

With this apparatus, Lange et al. (1977) have studied the binding of the substrate camphor to the bacterial cytochrome P-450. The rapid rate of association had previously prevented the direct measurement of the rate constant of temperatures above $0^\circ C$. However, it was possible to measure the reactions directly in a 1:1 (v/v) mixture of ethanediol and water at 0 and $-20^\circ C$. (In this case, however, the binding of camphor in the hydrophobic pocket is strongly influenced by the aqueous/organic medium; the reaction at $0^\circ C$ is 60 times slower in the ethanediol medium than it is in water. Such a large effect means that caution is necessary in applying conclusions drawn from experiments performed under such conditions to the normal situation.) The association and dissociation rate constants were measured [the latter by the rapid and irreversible
Rate-limiting step

\[ \text{H}_2\text{O} \rightarrow \text{Photosystem II} \rightarrow \text{PQ} \rightarrow f + ? \rightarrow \]

\[ \text{PC} \rightarrow \text{Photosystem I} \rightarrow \text{Fd} \rightarrow \text{FP} \rightarrow \text{NADP} \]

Scheme 2. Photosynthetic electron-transport chain

The boxes represent Photosystems I and II with their reaction-centre chlorophylls and associated pigments and electron-transport components, and the membrane-bound components including cytochrome \( f(f) \) between plastoquinone (PQ) and plastocyanin (PC). Abbreviations: Fd, ferredoxin; FP, flavoprotein (ferredoxin–NADP oxidoreductase).

trapping of unliganded Fe(III)-cytochrome \( P-450 \) with metapyrone] as a function of temperature, and the enthalpies and entropies of activation calculated. The rate constants were shown to depend on pH* in such a way as to suggest the involvement of an imidazole residue in substrate binding.

Chloroplast electron transport: behaviour of a complex membrane-bound system. A further example of the application of fluid solvent systems at subzero temperatures is provided by investigations on the electron-transport chain of the chloroplast (thylakoid) membrane. This contains two photochemical centres arranged in series and connected by a number of electron-transferring components (Scheme 2). In addition to oxidizing water and reducing a low potential acceptor (naturally, ferredoxin and then NADP*) the electron-transport chain is also capable of generating a proton gradient across the thylakoid membrane (which can then be used to phosphorylate ADP). [For a recent review see Bendall (1977).]

The rate-limiting step of the chain is the re-oxidation of plastoquinone situated between the two photosystems, which has a turnover time of about 10 ms at normal temperatures (Witt, 1975). Because of the high background absorbance due to light-scattering and the presence of pigments, the study of the kinetics of redox changes of individual components in this time range requires highly-specialized purpose-built equipment usually involving a repetitive-flash technique, where absorbance changes following a series of flashes (up to several thousand) are added together to improve the signal-to-noise ratio. Steady-state changes, on the other hand, can be studied on commercially available dual-wavelength spectrophotometers, which have a useful response time of about 1 s for this type of experiment. It would be very valuable to be able to use this type of apparatus for turnover studies, and the use of low temperatures provides a way of doing this.

Many reactions of chloroplasts can still occur at subzero temperatures in fluid mixed solvents (Hall & Arnon, 1962; Amesz et al., 1973; Cox, 1975a,b,c; Amesz & De Grooth, 1976). Electron transport through both photosystems to an artificial electron acceptor has been demonstrated down to \(-35^\circ\text{C}\) in a medium containing 1:1 (v/v) ethanediol/water (Cox, 1975a).

During steady-state illumination, components before the rate-limiting step, including the plastoquinone, are reduced and those after it, including cytochrome \( f \) and the reaction-centre chlorophyll of Photosystem I (pigment P700), are oxidized. Measurement of the reduction of cytochrome \( f \) and pigment P700 in the dark after illumination is therefore a way of studying the rate-limiting step. At \(-30^\circ\text{C}\) the half-time of pigment P700 reduction was decreased to 20 s, a decrease of more than \(10^3\) compared with the situation at normal temperatures (Cox, 1975b). Under these conditions the time course of reduction
could be accurately measured during a single light–dark cycle, by using the dual-wave-
length technique. It is noteworthy that the rates of reduction of both cytochrome $f$
and pigment P700 were increased by uncoupling agents, suggesting that the membrane
is sufficiently intact to be able to maintain a proton gradient under these conditions.

Haehnel (1973) had previously studied the reduction of cytochrome $f$ and pigment
P700 after illumination by using the repetitive-flash technique, and reported that cyto-
chrome $f$ did not show the kinetics expected from the reported redox potentials of the
various components and a simple series:

\[
\text{Plastoquinone} \rightarrow \text{cytochrome} f \rightarrow (\text{plastocyanin}) \rightarrow \text{pigment P700}
\]

which predicts a lag in cytochrome $f$ reduction until pigment P700 is mostly reduced.
By using low temperatures it was possible to confirm this observation, arguably with
greater precision, because of the superiority of the dual-wavelength technique over
the single-wavelength measurements used by Haehnel (1973). Both Haehnel (1973)
and Cox (1975b) found first-order kinetics for the reduction of cytochrome $f$ as well as
for pigment P700, differing by a factor of 3.

The arrangement of the electron-transport components in this part of the chain is still
subject to debate, and the discovery of an iron–sulphur centre in chloroplasts with a
redox potential between those of plastoquinone and cytochrome $f$ (Malkin & Aparicio,
1975) means that an extra component must be considered. A satisfactory model will have
also to take into consideration the two-electron character of the plastoquinol → plasto-
quinone reaction. However, the results at low temperatures join the increasing body of
evidence suggesting that this segment of the electron-transport chain may be more
complex than at first supposed. They also demonstrate the value of the low-temperature
approach in studying this type of complex membrane-bound multi-enzyme system.

Conclusion

Is the low-temperature approach really useful? With a few notable exceptions, the
results obtained so far using low temperatures have been mostly in the nature of
confirmations of what is already known; elegant in some cases, but not making any
really new contributions to biochemical knowledge. Until now the use of low
temperatures has also been confined to a small band of specialists. Partly this
may be because the technique has become surrounded by an unnecessary mystique,
partly from a commendable caution about the real value of results obtained under such
bizarre conditions.

Experience to date suggests that many systems can be made to function at subzero
temperatures with useful and sometimes dramatic decreases in rate. The examples dis-
cussed demonstrate that such decreases can allow completely new information to be
obtained, as well as allowing experiments which are already feasible to be performed
with greater ease or precision. Naturally the problem of artefacts produced by the
abnormal conditions must always be borne in mind; however, few if any techniques are
entirely free of the risks of producing misleading results, and used with caution this
approach is not significantly different from others.

The low-temperature approach has great potential in biochemistry. It deserves to be
more widely used.

(Northcote, D. H., ed.), vol 13, pp. 41–78, University Park Press, Baltimore
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