Biophysical Chemistry 24 (1986) 41-46
Elsevier

BPC 01052

SUBZERO-TEMPERATURE PRESERVATION OF REACTIVE FLUIDS IN THE UNDERCOOLED STATE

I. THE REDUCTION OF POTASSIUM FERRICYANIDE BY POTASSIUM CYANIDE

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Received 18th December 1985
Accepted 18th February 1986

Key words: Ferricyanide; Undercooling; Preservation; Reaction rate; Cryopreservation; Freeze concentration

Many reactions show enhanced rates at subzero temperatures due to freeze concentration. The reduction of potassium ferricyanide by potassium cyanide has been studied at subzero temperatures in both the undercooled and the frozen state. The pseudo-first-order rate constants calculated differ greatly from those in previous reports. A high degree of freeze concentration and supersaturation in frozen bulk solutions occurs. It has been clearly demonstrated that undercooled preservation provides a useful method for the long-term storage of reactive mixtures.

1. Introduction

Many reactions show greatly enhanced rates at subzero temperatures when partly frozen and this is widely accepted to be principally because of the increase in concentration of substrates during freezing [1]. Other factors which cannot be ignored include catalytic effects of ice crystals, greater proton mobility in ice than water, a favourable substrate-catalyst orientation caused by freezing, and a higher dielectric constant for water than ice [2-6].

The system studied in this experiment was a mixture of potassium ferricyanide ($K_3Fe(CN)_6$) and potassium cyanide, at low concentration, as commonly used to assay haemoglobin in blood [7]. As commercially used, the reagent is despatched by the manufacturer in the form of a solution which is stable above 0°C and is based on the formulation given by Drabkin and Austin [8]. On exposure to subzero temperatures, however, the ferricyanide is completely reduced to ferrocyanide [7]. At high concentrations and/or temperatures [9], or under reflux conditions [10], the reaction proceeds to give different products, as follows:
Under reflux:

$$2\mathrm{Fe(CN)}_6^{3-} + 2\mathrm{CN}^- + 2\mathrm{H_2O} \rightarrow 2\mathrm{Fe(CN)}_6^{4-} + \mathrm{HCN} + \mathrm{NH_3} + \mathrm{CO_2} \quad (1)$$

At high concentration:

$$2\mathrm{Fe(CN)}_6^{3-} + \mathrm{CN}^- + 2\mathrm{OH}^- = 2\mathrm{Fe(CN)}_6^{4-} + \mathrm{CNO}^- + 2\mathrm{H_2O} \quad (2)$$

The products of reaction 2 may be unstable at high temperatures and therefore the complete reaction would be the same as that shown in eq. 1, with the products of reaction 2 being intermediates of the total process. The mechanism for reaction 2 is thought to be [11,12]:

$$\mathrm{Fe(CN)}_6^{3-} + 2\mathrm{CN}^- = \mathrm{Fe(CN)}_6^{4-} + (\mathrm{CN})_2^-$$
$$(\mathrm{CN})_2^- + x\mathrm{H_2O} \rightarrow \mathrm{P} + \mathrm{CN}^-$$
$$\mathrm{Fe(CN)}_6^{3-} + \mathrm{P} \rightarrow \mathrm{Fe(CN)}_6^{4-} + \mathrm{Q}$$

where P and Q are undefined intermediates. K^+ does not participate in the reaction but may exert a catalytic effect [9].

Undercooling has already been shown to provide a viable method for storing cells and cell-free solutions at temperatures down to −40°C [13]. The objective of this study was to investigate whether under such conditions, i.e., subzero temperatures without freezing, enhancement of reaction rates takes place. Biological fluids, normally damaged by freezing or by detrimental reactions at refrigerator (chill) temperatures could therefore possibly be stored successfully at subzero temperatures in the undercooled state.

The reduction of ferricyanide by cyanide was chosen as a model system for this investigation because it can easily be stopped by warming the reaction mixture to > 0°C. The concentration of ferricyanide can then be measured spectrophotometrically.

2. Materials and methods

Equal volumes of potassium ferricyanide (1.21×10^{-3} M) and KCN (1.53×10^{-3} M) were mixed and used as a model system for the study of the effect of low temperature and freezing on reaction rates. A control sample was held at room temperature throughout the investigation and was periodically tested for the disappearance of ferricyanide. 3.5 ml samples of the reaction mixture were placed in 30 ml plastic Universal tubes (Northern Media Supply Ltd.) to facilitate rapid cooling. They were treated according to one of two protocols as follows:

(A) The samples were held in an ethylene glycol bath set at −20°C for 6 min after which crystallisation was initiated by striking the side of the tube. They were then placed in a freezer at −5, −20, −30 or −40°C.

(B) The samples were cooled in an ethylene glycol bath set at 20 degrees below the desired storage temperatures. Ice crystals were initiated at −5°C, by the same method as in A, and the bulk solution was placed back into the bath until the required temperature was reached. The samples were then transferred to a freezer set at the required storage temperature. This allowed a higher cooling rate than in protocol A to be attained. In order to achieve undercooling and prevent stray ice nucleation in the reaction mixture the water-in-oil droplet emulsion technique was used [14].

Emulsified samples were prepared from 1 ml of the aqueous reaction mixture dispersed in 10 ml mineral oil with a Polytron coaxial cylinder homogeniser. The emulsion was then stabilised by being placed in ice before subjection to a cooling process identical to that described for the bulk samples in A and B.

Emulsified and non-emulsified samples were periodically removed and warmed to room temperature in a water bath set at 35°C. The aqueous mixture was separated from the oil by centrifuging at 2400 rpm in an MSE Centaur 2 centrifuge for 15 min. A 0.6 ml sample of the aqueous reaction mixture was then carefully removed and placed in a cuvette; to this 0.18 ml distilled water was added. Its absorbance and that of a non-diluted sample of the non-emulsified reaction mixture was measured at 426 nm in a Perkin-Elmer P557 double-beam, dual-wavelength spectrophotometer [15]. A further sample of 1 M ferricyanide/1.25 M KCN mixture at room temperature was used to simulate the effect of freeze concentration.

The pH of a fresh non-reacted aqueous sample and a sample that had been allowed to react (by freezing) was measured. The temperature was monitored throughout the experimental procedures with a thermocouple immersed in the samples.

Emulsions consisted of 1 ml aqueous phase dispersed in 10 ml oil, therefore it would be difficult to detect any freezing of the aqueous phase in the emulsion by the rise in temperature associated with the release of latent heat. To overcome this, a Perkin Elmer DSC-2 calorimeter was used to establish the ice nucleation temperature of the aqueous phase in the emulsion.

The rate of reaction in a bulk solution was measured at 90 and 75°C using both a 1.21 and a 50 mM ferricyanide solution in the presence of excess KCN.

The effect of pH was studied at 75°C with 50 mM ferricyanide. The rates of reaction in a standard solution with pH 11, and a second solution with the pH adjusted to 10 by the addition of HCl, were also measured.

3. Results

Measurements of the change in absorbance with time for the frozen bulk and non-frozen undercooled samples are shown in figs. 1–3. At subzero temperatures in emulsion the rate was comparable to that of the control sample held at room temperature (figs. 2 and 3), variation in absorbance only being due to light scattering of residual oil in the aqueous solution. No change in ferricyanide concentration could be detected over extended periods (figs. 2 and 3). This was to be expected because cooling curves (fig.4) and DSC power-time curves indicated that the emulsions did not freeze at the experimental temperatures.

A pH difference of 0.5 pH units was observed between an unfrozen solution and a frozen reacted solution. A difference was also observed between the reaction rates of 0.05 M ferricyanide solutions at pH 11 and 10, with rate constants (k) being 8×10^{-4} and 1.28×10^{-3} min^{-1}, respectively.

The cooling curves in fig. 4, when compared to the times of rapid changes in absorbance in fig. 1, show that at −12°C the rate is low enough for the solution to cool to this temperature before most of the reaction has taken place. Rates could therefore be accurately calculated. For samples cooled below −20°C the reaction was complete before the solution had reached a constant temperature. An accurate calculation of k could not therefore be made.

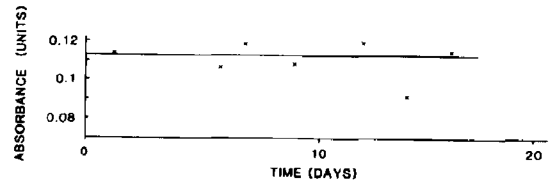

Fig. 2. Change in absorbance of ferricyanide with time in an undercooled emulsified solution at −12°C. The scatter of points is due to the turbidity caused by the residual oil in the aqueous phase of the broken emulsion.

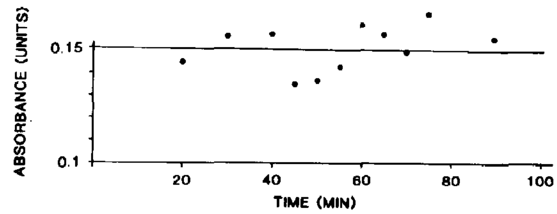

Fig. 3. Change in absorbance of ferricyanide with time in an undercooled emulsified solution at −20°C.

More rapid cooling was achieved by placing the samples in an ethylene glycol bath kept below the required temperature (protocol B). When the samples reached the required temperature, they were rapidly transferred to a freezer set at that temper-

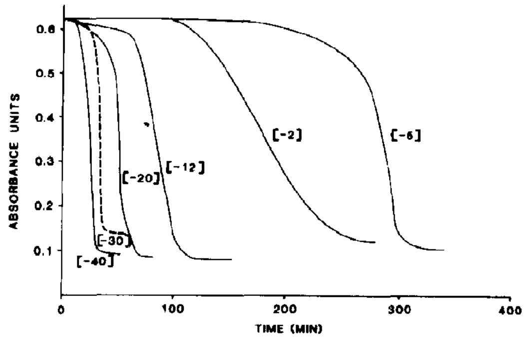

Fig. 1. Change in absorbance of ferricyanide with time in a bulk solution at temperatures from −2 to −40°C.

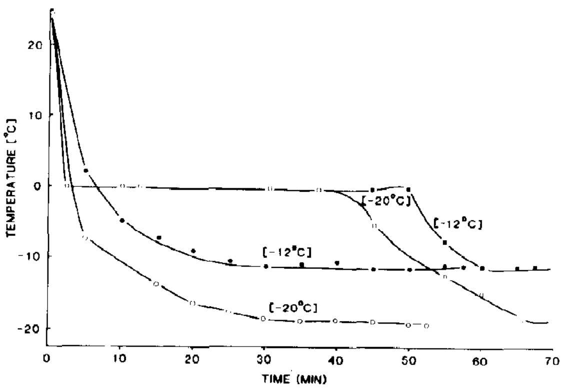

Fig. 4. Cooling curve for emulsified (○) and bulk (□) solutions.

ature and rate measurements were made. This enabled an accurate rate at −20°C to be measured, but cooling was not sufficiently rapid for rates at −30 or −40°C to be determined.

Since the reaction is pseudo-first order with respect to ferricyanide when KCN is in excess [9], rate constants (*k*) can be measured. An Arrhenius plot (fig. 5) shows a rapid increase in rate when freezing takes place. Acceleration values of up to 40 500 are achieved, as shown in table 1, where the acceleration value (*s*) is the ratio of the observed rate constant under conditions of freeze concentration to the hypothetical Arrhenius rate constant at the same temperature but in the undercooled state.

In most kinetic studies with frozen solutions, after an initial increase in reaction rate due to freezing, a decrease in rate has been observed [6], *s* then remaining constant or decreasing with decreasing temperature. For the reaction described in this study a decrease in *s* could not be observed.

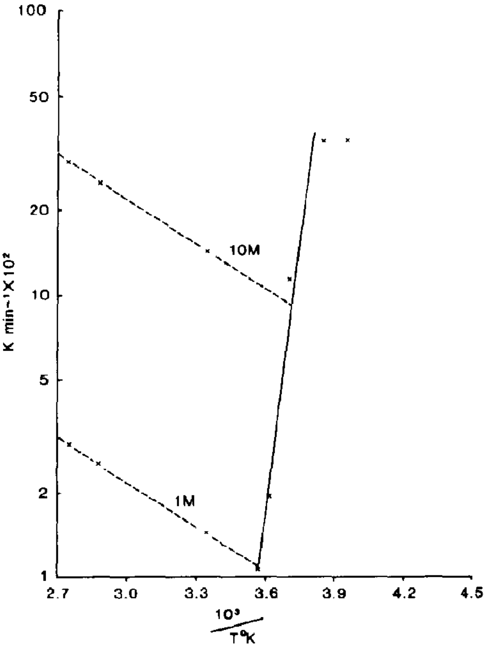

Fig. 5. Plot of k_{obs} (min^{-1}) vs. $1/T$ for the freeze-concentrated reaction mixtures, and calculated Arrhenius plots (- - - -) based on measurements at elevated temperatures (see text).

Table 1

Effect of freezing on the rate constant (*k*)

Storage temperature (°C)	Observed rate constant (min^{-1})	Predicted rate constant (min^{-1})	Acceleration value (*s*)
−2	1.05×10^{-2}	1.18×10^{-5}	890
−5	1.95×10^{-2}	1.12×10^{-5}	1 741
−12	11.50×10^{-2}	1.00×10^{-5}	11 500
−20	36.00×10^{-2}	8.90×10^{-6}	40 449

Measurement of the rates at 90 and 75°C in a 0.05 M solution gave *k* values of 1.48×10^{-3} and 1.27×10^{-3} min^{-1}, respectively. As the reaction is pseudo-first order, *k* is proportional to the concentration. Values for the rate at higher concentrations can therefore be estimated by simple proportionality, provided that the ratio $Fe(CN)_6^{3-}/CN^-$ remains constant.

Idealised Arrhenius plots based on the measured rate constants at 75 and 90°C for 0.05 M solution were used to estimate the concentration of ferricyanide at various temperatures when freeze concentrated. Fig. 5 shows estimated Arrhenius plots for 1 and 10 M ferricyanide solutions. Extrapolation of these plots to subzero temperatures indicates that the bulk solution of initial concentration 6.05 mM was freeze concentrated to 1 M with respect to ferricyanide at −1°C and to 10 M at −13°C.

4. Discussion

As shown in fig. 1, the rapid change in absorbance was preceded by a plateau phase, the duration of which was related to the temperature of the freezer into which the solution was placed. This phase corresponds to the isothermal plateau caused by the low rate of dissipation of the latent heat of freezing. Once all the heat had been dissipated, the temperature of the sample dropped and the reaction began. The reaction did not proceed rapidly during the isothermal plateau because at this temperature (−0.6°C) the freeze concentration of the reactants was insufficient to accelerate the reduction of ferricyanide to a marked extent. The highest subzero temperature at which a significant increase in rate could be measured was −2°C.

From fig. 1 it can be seen that at −2°C the sample reacted before, but not more rapidly than the sample kept at −5°C. This was because the entire reaction was carried out in an ethylene glycol bath at −2°C. The better heat dissipation properties of this bath meant that the lag phase was shorter, so that the reaction commenced earlier than in the −5°C sample which was placed in the freezer immediately after ice crystal initiation.

The calculation of k showed an increase in the rate of reaction with a decrease in temperature from −2 to −12°C, principally caused by an increase in concentration of the reactants as ice crystallises out. This indicates that k is far more dependent on concentration than on temperature; most reactions in frozen reactive mixtures show a decrease in rate in this temperature range [6]. In all frozen solutions, however, it is impossible to measure the effect on k of temperature alone, as freeze concentration always has an effect. Eventually, at very low temperatures, the reaction will show a decrease in rate as it reaches a point where a limiting freeze concentration has been reached. The rate of ferricyanide reduction makes it unlikely that this temperature is accessible to experiment. Extremely rapid cooling would be needed to attain this temperature before the reaction was complete.

At all temperatures the reaction between ferricyanide and cyanide in bulk frozen solutions occurred far more rapidly than expected from the data of Weatherburn and Logan [7]. Their results gave complete conversion at −12, −25 and −78°C, as 7 h, 2 h and 107 s, respectively. These results are entirely different from those found in this study, although their values of 7 h at −12°C, and 2 h at −25°C could have been obtained if the solution had been allowed to supercool. Since the authors do not give any indication of having used ice crystal seeding, this may well have been the case. At −78°C a reaction time of 107 s is reported but no mention is made of the technique used to quench the sample down to −78°C or the method used to determine the rate. As 5 ml samples in ‘screw capped tubes’ were used, it seems unlikely that a cooling rate high enough to reach −78°C before the reaction reached completion could have been achieved.

Measurements of pH changes during the reaction in a frozen solution indicated a reduction of 0.5 pH units after the reaction had reached completion. At 75°C a reduction in reaction rate was found if the pH of the solution was lowered by 1 pH unit. These results suggest that OH^- is being removed during the reaction and that reaction 2 is taking place. In a frozen solution any water produced in reaction 2 would freeze, driving the reaction to the right until all the ferricyanide had been reduced. Tests for ferricyanide ions in a reacted mixture were negative [7], suggesting this is indeed the reaction path and that freeze concentration is the driving force.

Freeze concentration of most second-order reactions results in an enhancement of rate, as calculated by Pincock and Kiovsky [16]:

$$\frac{d[A_t]}{dt} = -k[A_u][B_u]\frac{V_u}{V_t} \qquad (3)$$

where $[A_u]$, $[B_u]$ are the concentrations of the reactants in the unfrozen portion of the partially frozen solution, $[A_t]$ the concentration of A if the solution is not frozen, and V_u and V_t the volumes of unfrozen solution in the unfrozen and partially frozen solution, respectively. As the reaction of ferricyanide and KCN is pseudo-first order with respect to ferricyanide at any temperature when KCN is in excess, eq. 3 can be simplified:

$$\frac{d\left[Fe(CN)_6^{3-}\right]}{dt} = -k\left[Fe(CN)_6^{3-}\right]_u\frac{V_u}{V_t}$$

The solubility of ferricyanide in water is 33 g/100 ml at 25°C to give an approx. 1 M solution. From the extrapolation of the Arrhenius plot for a 1 M solution it is seen that this concentration is reached by freezing at −1°C. At lower temperatures the rate of reaction continues to increase, implying that the solution becomes supersaturated. A hypothetical 10 M plot, shown in fig. 5, indicates that a high degree of supersaturation must occur in the freeze-concentrated mixtures. It is relevant to note that no precipitation of reactants or products could ever be observed during the experiments, irrespective of the conditions employed.

Below −20°C the rate of reaction could not be accurately determined because sufficiently rapid cooling could not be achieved. The reaction rate at −30°C was, however, found to be significantly higher than that at −12°C. It appears, therefore, that a very high degree of supersaturation occurs. High levels of supersaturation are not uncommon in frozen or dehydrated solutions [17], so that the 47-fold supersaturation which occurs at −20°C is not unusual.

Emulsification of the reaction mixture prevented freezing and hence freeze concentration. At storage temperatures of −20°C there was no indication of any rate enhancement over that found at room temperature. The results of this investigation indicate that reaction rates in undercooled solutions follow those predicted by the Arrhenius equation. Depending on the activation energy, this could result in significant rate reductions enabling reactive fluids which at present can only be stored for limited periods under conditions of refrigeration, but are destroyed by freezing, to be stabilised at subzero temperatures for weeks or months.

Acknowledgements

We thank the AFRC for the grant to H.D. We also thank Sheila Mathias for her critical but constructive reading of the manuscript.

References

1 O. Fennema, in: Water relations of foods, ed. R.G. Duckworth (Academic Press, London, 1975) p. 539.
2 H.E. Alburn and N.H. Grant, J. Am. Chem. Soc. 87 (1965) 4174.
3 T.C. Bruice and A.R. Butler, J. Am. Chem. Soc. 86 (1964) 4104.
4 N.H. Grant and H.E. Alburn, Science 150 (1965) 1589.
5 N.H. Grant and H.E. Alburn, Arch. Biochem. Biophys. 118 (1967) 292.
6 N.H. Grant, D.E. Clark and H.E. Alburn, J. Am. Chem. Soc. 83 (1961) 4476.
7 M.W. Weatherburn and J.E. Logan, Clin. Chim. Acta 9 (1964) 581.
8 D.L. Drabkin and J.H. Austin, J. Biol. Chem. 98 (1932) 719.
9 C.H. Bandford and L.F.H. Tipper, Comprehensive chemical kinetics, vol. 7 (Elsevier, Amsterdam, 1972) p. 410.
10 J.N. Friend, Inorganic chemistry, vol. 9 (Griffin & Co. London, 1921)
11 J.S. Littler and I.G. Soyce, J. Chem. Soc. (1965) 2545.
12 A.W. Anderson, J. Phys. Chem. 56 (1952) 858.
13 S.F. Mathias, F. Franks and R.H.M. Hatley, Cryobiology 22 (1985) 537.
14 F. Franks, S.F. Mathias, P. Galfre, S.D. Webster and D. Brown, Cryobiology 20 (1983) 298.
15 E. Racker, Biochim. Biophys. Acta 19, (1952) 577.
16 K.R. Diller, Cryobiology 22, (1985) 268.
17 R.E. Pincock and T.E. Kiovsky, J. Am. Chem. Soc. 88 (1966) 4455.
18 C. Gazeau, Can. J. Bot. 63 (1985) 663.