The Chemical Constitution of Compounds Which Protect Erythrocytes against Freezing Damage

T. NASH

From the National Institute for Medical Research, Mill Hill, London, England. Mr. Nash's present address is the Medical Research Council, Air Pollution Research Unit, St. Bartholomew's Hospital, London, England

ABSTRACT Eleven simple neutral water-miscible compounds were tested for protective action against freezing damage to human red blood cells. All the compounds penetrated the cells at room temperature without damage, but only four, N-substituted amides, were active. These results are considered together with previously published work on freezing protection by other low molecular weight solutes. The affinity of the compounds for water is gauged in two independent ways, and correlates well with protective ability. The chemical constitutional factors responsible for high affinity for water are discussed. It appears that basic character is most important.

The neutral solutes which can protect living cells such as spermatozoa and red blood cells against freezing damage belong to various chemical species. They include glycerol (Polge, Smith, and Parkes, 1949), other hydroxylic compounds and acetamide (Lovelock, 1954), dimethyl sulfoxide (Lovelock and Bishop, 1959), and pyridine N-oxide (Nash, 1961). The present work is an attempt at finding a chemical constitutional basis for such protection.

With many living cells, damage due to freezing and thawing can be satisfactorily explained by reference to the rise in concentration of salts and possibly of other normal cell solutes which must occur when pure water separates out as ice inside the cells (Lovelock, 1953). The question is considered in detail in a recent monograph (Smith, 1961) and it is clear that there is a mass of quantitative evidence, due mainly to Lovelock, in favour of the salt damage theory which is not matched by any other. According to this theory, neutral solutes such as glycerol protect against freezing damage by penetrating inside the cell and lowering the concentration of salt in equilibrium with ice at any temperature below freezing (Lovelock, 1954). The molar concentration of solute must therefore be fairly high and there is consequently an upper limit to the permissible molecular weight, in the...
region of 150. The main requirements are penetrative ability, resistance to salting out at low temperatures, and lack of toxicity.

Red blood cells are used in the present work, but cautious generalization of the results to other cells is permissible. There is also, from the above, a close relation between damage due to freezing and that due to drying. Again, resistance to heat is often bound up with the possibility of non-lethal dehydration. A solute which can protect red cells against freezing damage may therefore be expected to have some application in the protection of many kinds of cells against adverse physical conditions.

**Water Solubility**

Apart from acetamide, the known good protectors are completely miscible with water, and it is reasonable to assume that protective action is connected with affinity for water. Non-electrolytes (neutral solutes) dissolve in water by forming hydrogen bonds with it, polarity as such not being important (Ewell, Harrison, and Berg, 1944). The factors which control the water-binding capacity of various groups have been discussed (Nash, 1962) and the relevant points are now summarised.

Since water is extensively hydrogen-bonded itself, solution is a competitive process, and can take place only if the solute-water bonds are at least as strong as the water-water bonds. In pure water, molecules must act equally as donors and acceptors, but most neutral solutes are hydrogen acceptors; i.e., they dissolve by means of lone-pair electrons on oxygen or nitrogen which coordinate the hydrogen of water (Coulson, 1957). The strength of the bond so formed depends first on the degree of localization of the lone pairs concerned, and second on the sign and magnitude of mesomeric charge transfer to the atom which bears them. Conjugation of this polar atom affects both localization and charge transfer, and is therefore critical in deciding whether the acceptor activity is strong enough to take the compound into solution, and to what extent.

The phenols are the only common (neutral) pure hydrogen donors, dissolving by forming hydrogen bonds with the water oxygen only, and not appreciably with the water hydrogen. They are also more physically toxic than is indicated by their chemical potential (Ferguson, 1939). The alcohols (including glycerol, etc.) on the other hand are hydrogen acceptors, as are the amides, but both kinds of compound have a slight degree of donor character which can become noticeable in the absence of water or other donor.

**Quantitative Assessment of Solubility**

The relative “solubility” of compounds, all of which are completely miscible with water, can be found indirectly by considering higher homologues, only
some of which can be miscible. An accurate comparison (Nash, 1962) requires the use of the parachor (Sugden, 1924), but for the present purpose it is sufficient to find the number of additional methylene groups in the lowest homologue not completely miscible with water. For example, methanol, ethanol, and n-propanol are all miscible with water, but n-butanol is not. The molecule of methanol is roughly equivalent in bulk to two methylene groups. Its solubility or "hydrophilic strength" (HS) can then be written as a fraction, \( \frac{2}{n} \), the numerator being the number of methylene groups which can be added before miscibility with water is lost, and the denominator the number of such groups equivalent in bulk to the original compound. For propanol, HS would be \( \frac{9}{n} \), and for ethanol \( \frac{1}{n} \).

Selection of Compounds for Testing

The number of possible neutral solutes is limited, as far as protective action is concerned, since those of high molecular weight or low solubility can be ignored. There also seems to be little loss in generality if attention is confined to liquids or compounds which melt at only slightly above room temperature. An important measurement which can be made on these but not on crystalline solids is that of the temperature change which occurs on mixing with water. The first fifteen compounds listed in Table I are representative of most of the different classes of polarity and are all neutral, water-miscible liquids of low molecular weight. The hydrophilic strength (HS) is based on a survey of published solubility data and, when necessary, the synthesis of model compounds and direct measurement of solubility (Nash, 1962). There is an uncertainty of one methylene group in the figure for methyl formamide and in that for glycerol, while that for formamide is in a sense fictitious since any kind of substitution must alter the molecule considerably. With other compounds, having at least one methyl or methylene group already present for starting a homologous series, this difficulty does not arise.

METHODS AND MATERIALS

Thermal Measurements

Because of the complicated nature of aqueous solutions, two measurements were made on each compound, one of the temperature change \( T(eq) \) which occurred on adding an equimolar amount of water, and a second of the temperature change \( T(dil) \) which occurred on making up a 2 M solution. For \( T(eq) \), 0.2 mole of the compound in a large test tube fitted with a thermometer was allowed to reach thermal equilibrium in a beaker of distilled water. It was then removed, the outside dried quickly, and 0.2 mole (3.6 ml) of the water added with stirring. The maximum rise or fall in temperature was recorded. \( T(dil) \) was found in a similar manner, except that a larger tube was used and 100 ml of water was added. With glycol only 0.1 mole, and with glycerol only 0.067 mole, was used as it was felt that the hydroxyl groups
should be considered individually. No correction was made for the thermal capacity of the vessels, but the same ones were used throughout so that the figures are comparable.

For the two solid compounds, acetamide and urea, the temperature change recorded is that found when equal volumes of water and of saturated solution (20°C) are mixed.

**Freezing Experiments**

Lovelock's (1954) procedure was followed. Suspensions of red cells were made up in buffered saline containing various concentrations of solute, and allowed to stand for 2 minutes at room temperature. The mixtures were made up in thin walled glass tubes only 0.7 cm in diameter, and required thorough shaking to become homogenous. The tubes were immersed in acetone-methanol freezing mixture, brought to the required temperature by adding solid carbon dioxide, for 15 minutes. They were then thawed in water at 38°C and spun down. Haemolysis was estimated colorimetrically in the supernatant, a control tube in every six having no added solute. Readings of haemoglobin concentration for the other five tubes were expressed as a percentage of that for the control.

Compounds were first tested at −79°C, and if haemolysis was complete for solute concentrations up to 3 M no further tests were done and the compound was classed as non-protecting. If haemolysis was not complete tests were done at various higher temperatures up to −20°C.

Pyridine N-oxide (Aldrich) was purified by dissolving in water, passing through charcoal, and dehydrating in a vacuum evaporator. Ethylene oxide (B.D.H.) and dimethyl sulfoxide (Baker Analyzed) were used without treatment. Other compounds were distilled at reduced pressure, and a central cut taken. Citrated human blood was stored at 4°C, for not longer than 5 days before use. Twice washed red cells were used on the day of preparation.

**RESULTS**

The results of the thermal measurements are listed in Table I, together with the hydrophilic strength as defined above, and the protective ability. The latter was already known for dimethyl sulfoxide, pyridine N-oxide, acetamide, glycol, and glycerol. Four simple N-substituted amides were also found to be good protectors, and these new results are given in detail in Fig. 1. It may be seen that 3 M solutions of these compounds afforded complete protection except for methyl formamide, which allowed a small amount of haemolysis at the lowest temperature used.

Similar sets of curves were obtained for dimethyl sulfoxide and for two equimolar mixtures, dimethyl formamide/glycerol and dimethyl formamide/dimethyl sulfoxide. The plots for dimethyl sulfoxide lay between those shown in the figure for the two dialkylamides, while the protective action of the components of the mixtures seemed to be additive, within experimental error.
Penetration and Haemolysis at Room Temperature

Solutions of the compounds, including acetamide and urea, were not haemolytic in 0.16 M saline at concentrations up to 2 M for 10 minutes. The most haemolytic were butyrolactone and acetonitrile, which lysed cells in 10 minutes at 2.5 M. In a further set of experiments red cells were added to 1 M solutions of the compounds in distilled water. Lysis occurred within 1 minute for all the compounds mentioned in Table I, including urea and acetamide. No lysis, and presumably no penetration, occurred with the salt-like non-protecting compound trimethylamine N-oxide.

Formation of "Ice" above Zero

A 20 per cent solution of ethylene oxide in water crystallized above zero, and was not free of solid until warmed to +10.5°C. Tetrahydrofuran also caused

<table>
<thead>
<tr>
<th>Compound</th>
<th>HS</th>
<th>Protection</th>
<th>$T_{(eq)}$</th>
<th>$T_{(dil)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethyl sulfoxide</td>
<td>4/4</td>
<td>Complete*</td>
<td>+15</td>
<td>+7.6</td>
</tr>
<tr>
<td>Dimethyl acetamide</td>
<td>3/6</td>
<td>Complete</td>
<td>+14</td>
<td>+8.6</td>
</tr>
<tr>
<td>Dimethyl formamide</td>
<td>3/5</td>
<td>Complete</td>
<td>+13</td>
<td>+6.3</td>
</tr>
<tr>
<td>Pyridine N-oxide</td>
<td>6+/6</td>
<td>Partial‡</td>
<td>+13</td>
<td>+6.3</td>
</tr>
<tr>
<td>Methyl acetamide</td>
<td>2/5</td>
<td>Complete</td>
<td>+10</td>
<td>+5.3</td>
</tr>
<tr>
<td>Methyl formamide</td>
<td>~3/4</td>
<td>Good</td>
<td>+8</td>
<td>+2.9</td>
</tr>
<tr>
<td>Ethylene glycol</td>
<td>4/4</td>
<td>Complete§</td>
<td>+7</td>
<td>+1.4</td>
</tr>
<tr>
<td>Glycerol</td>
<td>~5/6</td>
<td>Complete∥</td>
<td>+5</td>
<td>+0.6</td>
</tr>
<tr>
<td>Tetrahydrofuran</td>
<td>0/5</td>
<td>None</td>
<td>+2</td>
<td>+4.0</td>
</tr>
<tr>
<td>Acetone</td>
<td>0/4</td>
<td>None</td>
<td>Zero</td>
<td>+3.5</td>
</tr>
<tr>
<td>Ethylene oxide</td>
<td>1/3</td>
<td>None‡</td>
<td>-1</td>
<td>+3.5</td>
</tr>
<tr>
<td>Formamide</td>
<td>(3/3)</td>
<td>None§</td>
<td>-2.5</td>
<td>-0.8</td>
</tr>
<tr>
<td>2-Pyrrolidone</td>
<td>1/6</td>
<td>None</td>
<td>-2.5</td>
<td>+3.6</td>
</tr>
<tr>
<td>Butyrolactone</td>
<td>1/6</td>
<td>None</td>
<td>-3</td>
<td>+0.6</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>0/3</td>
<td>None</td>
<td>Zero</td>
<td></td>
</tr>
<tr>
<td>Acetamide¶</td>
<td>—</td>
<td>Good§</td>
<td>+1.3</td>
<td></td>
</tr>
<tr>
<td>Urea¶</td>
<td>—</td>
<td>None</td>
<td>-1.2</td>
<td></td>
</tr>
</tbody>
</table>

* Lovelock and Bishop (1959).
‡ Complete down to −40°C (Nash, 1961).
§ Lovelock (1954).
∥ Polge, Smith, and Parkes (1949).
¶ Solids (see text).
Figure 1. The haemolysis when red blood cells suspended in 0.16 M saline containing various concentrations of amide are frozen. The concentration is shown on each curve. In all the experiments the cells were frozen for 15 minutes at the temperature indicated. MF, MA, methyl formamide and methyl acetamide. DMF, DMA, dimethyl formamide and dimethyl acetamide.

separation of ice above zero, but ethyl ether, dioxane, acetone, dimethyl acetamide, and dimethyl sulfoxide depressed the freezing point of water in the normal manner. Red cells were not lysed if “frozen” above zero in the presence of ethylene oxide, but the compound did not protect against lysis at $-79^\circ$C.
DISCUSSION

In order to obtain the heat of mixing from $T(eq)$ a knowledge of the specific heat of the mixture would be required. The few values available in published tables are around 0.7 to 0.75 and it is clear that for the present purpose, namely, tabulation of the compounds according to the amount of heat evolved on mixing with water, the figures for temperature rise are adequate. It may be seen that there is a good correlation among protective ability, the hydrophilic strength $HS$, and $T(eq)$, but not $T(dil)$. Solutions of polar non-electro-
lytes in water are among the most complex of binary systems, and some of
the difficulties of a thermodynamic treatment have been discussed by Butler
(1946). It is not possible to assign any special significance to $T(dil)$ or $T(eq)$
in relation to protective action except perhaps that it could not have been
foreseen that the correlation would be with $T(eq)$ rather than with $T(dil)$,
**i.e.** with the partial molar heat of solution of water in the compound, rather
than with that of the compound in water. This result is possibly indicative of
the fact that the liquid phase of frozen solutions must in the last critical
stages have less water than solute.

**Basic Character of Protectors**

As mentioned above, the solubility of all the compounds mentioned in the
table (except possibly urea, see below) is due to the acceptor properties of
their lone-pair electrons (basic function) and not to active hydrogen (acidic
function). The compounds are arranged in order of decreasing $T(eq)$ because
that seemed to show up the correlations to best advantage. Basic character
and hydrophilic strength (HS) similarly decrease down the table. Dimethyl
sulfoxide is known to form the sulfoxonium cation in strong acid solution,
while pyridine $N$-oxide has a measurable $pK$ of 1.7 and is possibly too basic
to be entirely non-toxic. With regard to the amides, formamide is a non-
protector, but progresses to a complete protector with successive methylation
of each of its three hydrogens. These changes are no doubt associated with
increase in basic strength and hydrogen-bonding ability on methylation.
Pyrrrolidone is not anomalous in this respect, since the increase in basic
strength on internal alkylation is opposed by the known increase in acidic
character which carbonyl compounds are known to undergo on cyclization;
**e.g.**, dimeron and barbituric acid. It is interesting to note, however, the close
resemblance in behaviour between pyrrrolidone and butyrolactone, showing
that the amide hydrogen in the former cannot be playing much part in
solubility.

Ethylene oxide and tetrahydrofuran have only a moderate affinity for
water, and their behaviour in solution seems to be governed by steric factors
as well as by hydrogen bonding, since the solids which separated out above
zero were almost certainly clathrate ices of the kind discussed by Pauling
(1961) in relation to the action of gaseous anaesthetics. The vapour of tetra-
hydrofuran was in fact found to induce long lasting but completely reversible
anaesthesia in the mouse.

Acetamide and urea are solids, so that no figures for HS or $T(eq)$ can be
given which can be compared with the others. However, the temperature
changes on diluting the saturated solutions are not inconsistent with the
general trend of the others, being positive for acetamide and negative for
urea. It is suggested that the various internal interactions of the three polar
groups of urea result in a decreased ability for external interaction with the solvent; it is also possible that in aqueous solution the excessive donor character of one of the hydrogens reduces the basicity. Some protective ability should, however, be shown by N-methylated derivatives, and also by N-methyl pyrrolidone.

I am indebted to Dr. T. S. Work and to Dr. Audrey U. Smith for many useful suggestions during the preparation of this paper, and to Dr. K. A. Bisset for supplying blood.

Received for publication, April 23, 1962.

REFERENCES

LOVELOCK, J. E., 1953, Biochim. et Biophysica Acta, 10, 414.
Nash, T., 1962, data to be published.