THE FROZEN CELL

A Ciba Foundation Symposium

Edited by
G. E. W. WOLSTENHOLME
and
MAEVE O'CONNOR

J. & A. CHURCHILL
104 GLOUCESTER PLACE, LONDON
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First published 1970

With 115 illustrations

Standard Book Number 7000 1445 4

Printed in Great Britain

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The Ciba Foundation

The Ciba Foundation was opened in 1949 to promote international cooperation in medical and chemical research. It owes its existence to the generosity of CIBA Ltd, Basle, who, recognizing the obstacles to scientific communication created by war, man's natural secretiveness, disciplinary divisions, academic prejudices, distance, and differences of language, decided to set up a philanthropic institution whose aim would be to overcome such barriers. London was chosen as its site for reasons dictated by the special advantages of English charitable trust law (ensuring the independence of its actions), as well as those of language and geography.

The Foundation's house at 41 Portland Place, London, has become well known to workers in many fields of science. Every year the Foundation organizes six to ten three-day symposia and three to four shorter study groups, all of which are published in book form. Many other scientific meetings are held, organized either by the Foundation or by other groups in need of a meeting place. Accommodation is also provided for scientists visiting London, whether or not they are attending a meeting in the house.

The Foundation's many activities are controlled by a small group of distinguished trustees. Within the general framework of biological science, interpreted in its broadest sense, these activities are well summed up by the motto of the Ciba Foundation: *Consocient Gentes*—let the peoples come together.

Preface

THIS meeting originated from Dr John Farrant's suggestion of a symposium to discuss the precise effects of different freezing or cooling procedures and different concentrations of protective compounds on physical parameters in biological systems. The aim was to try to relate the physical changes to the biological properties necessary for cell viability and to learn more about the basic mechanism, or mechanisms, of freezing injury and protection.

Dr Farrant, Dr Peter Mazur, and their colleagues gave invaluable advice during the organization of the meeting, and the Ciba Foundation is most grateful to them for all their help. We are also indebted to Dr Farrant for much kind assistance with editorial difficulties. Dr Peter Mazur's firm and persuasive direction as chairman helped to throw many of the questions into sharper focus.

During the meeting the Clinical Research Centre Laboratories at the National Institute for Medical Research invited the participants, together with members of the British Cryogenic Council and other guests, to attend an afternoon of demonstrations and a garden party at Mill Hill. Dr Audrey Smith in this way contributed a valuable part to the symposium, although she could not manage to attend the actual proceedings. Also, very appropriately, Sir Alan and Lady Parkes acted as hosts for the Ciba Foundation at the main social function held at 41 Portland Place.

CHAIRMAN'S OPENING REMARKS

PETER MAZUR

ALL of us here are convinced, I am sure, that the responses of cells and macromolecules to subzero temperatures are matters of interest and of biological significance. Perhaps the best evidence that this view is not over-parochial is the fact that Dr Wolstenholme and the staff of the Ciba Foundation have organized this present symposium. The original impetus for the meeting came from Dr John Farrant, and we are especially indebted to him, to Dr Audrey Smith, and to Dr Wolstenholme for bringing it to fruition.

Whether by accident or design, our discussion is occurring on the 20th anniversary of the year that Polge, Smith and Parkes (1949) published their findings that glycerol enables mammalian sperm to survive slow freezing. The impact of that paper and its immediate successors on the field of low-temperature biology is difficult to exaggerate. It is perhaps equalled only by Lovelock's detailed and critical analysis of freezing injury in the human red cell, published some four years later. As you recall, Lovelock concluded that freezing and thawing cause haemolysis because freezing subjects cells to concentrations of electrolyte above mole fraction 0·014 (~ 0·8 M), and because thawing subjects them to dilution. He further concluded that glycerol and dimethylsulphoxide prevent haemolysis by preventing the electrolytes from concentrating to the critical value, and that this action is a consequence of the colligative properties of solutions. The total mole fraction of solute in a partially frozen solution is determined by temperature. If all the solutes are electrolytes, then the required mole fraction will consist entirely of electrolytes, but if a non-electrolyte such as glycerol is present, the concentration of electrolyte will be reduced, and the extent of reduction at a given temperature will be approximately proportional to the osmolar ratio of glycerol to electrolyte.

Lovelock stated that only low-molecular weight hydrophilic solutes with low eutectic points could protect cells, because only such solutes could yield solutions of high molar concentration at sufficiently low temperatures. He also stated that to be protective a solute must permeate a cell, for otherwise it could not prevent a rise in intracellular electrolyte during freezing.

So persuasive were the findings of Smith, Lovelock, and their colleagues that other investigators have tended to assume that the freezing procedures developed for sperm are also optimal for cells in general, and they have tended to assume that Lovelock's explanation of freezing injury and protection holds for cells in general.

The central question I would like to pose for our consideration is: to what extent are these assumptions valid ? Let me elaborate:

(1) Lovelock's explanation of freezing injury is detailed and specific. But slow freezing not only causes electrolytes to concentrate, it also causes the removal of water, the osmotic shrinkage of cells, and a decrease in the spatial separation of macromolecules. Some investigators, notably Meryman (1968) and Levitt (1966), believe that freezing injury is due more to these events than to the concomitant concentration of electrolytes.

(2) However, Lovelock's, Meryman's, and Levitt's theories agree in ascribing most freezing injury to a single cause, and in ascribing this single cause to changes in properties of solutions brought about by dehydration. Injury is not a result of ice formation *per se*. On the other hand, some of us believe that there are *at least* two factors responsible for injury, and that one of these is in fact ice formation within the cell. Furthermore, one of our speakers, Dr Brandts, has strong theoretical and experimental evidence for the existence of a third potentially injurious factor, namely, lowered temperature itself.

(3) These questions as to causes of injury have their counterpart in the protection of cells. Do all additives protect by a single mechanism, and, if so, is it by the colligative mechanism proposed by Lovelock?

(4) Related to questions of mechanisms of injury and of protection are questions as to the site and nature of the lesions. I believe that several speakers will present evidence to strengthen the growing suspicion that the chief targets of injury are membranes. If so, it then follows that additives probably protect cells by somehow protecting their semipermeable membranes.

Cells contain both surface and internal membranes. Are they both susceptible to injury? Apparently so if additives must permeate cells in order to protect them. But is this long-held assumption correct? Is permeation by an additive obligatory for protection? The main affirmative evidence again appears to be that provided by Lovelock. He showed that fewer red cells survive freezing in non-permeating additives than survive in permeating additives. However, what has been generally ignored is that he also found that many more red cells survived freezing in non-permeating additives than survived in the absence of any additive. Furthermore, Rapatz and Luyet (1963) and Rapatz, Sullivan and Luyet (1968) have reported that human red cells and bovine red cells respond similarly to freezing in glycerol in spite of the fact that the latter are many times less permeable to glycerol. Some of these points are summarized in Table I. Other evidence on this matter with other cells will be presented by several speakers.

TABLE I

RELATION BETWEEN PERMEATION OF GLYCEROL AND RECOVERY OF RED CELLS AFTER FREEZING

Cell	*Time for permeation of glycerol at*		*Recovery (%) of cells frozen slowly to* -20 °C *or below after exposure to indicated concentrations of glycerol*				
						14%, + Cu^{++} at	
	20 °C	0 °C	0%	10%, 30 sec at 0 °C	10%, 10 min (?) at 20 °C	0 °C	40 °C
Human	1 min*	4 min*	2†	78‡	96†	72*	97*
Bovine	$\sim$30 min§	—	0‖	75‡	92‖	—	—

* Lovelock (1953).
† Rapatz, Sullivan and Luyet (1968).
‡ Lovelock and Bishop (1959).
§ Jacobs (1931).
‖ Rapatz and Luyet (1963).

The question of the relation between the ability of a solute to permeate and its ability to protect against freezing injury is, it seems to me, a vital one. The answer is essential to understanding the fundamental causes of injury and it may well be essential to the successful freezing of cellular aggregates.

REFERENCES

JACOBS, M. H. (1931). *Ergebn. Biol.*, **7,** 1. [Cited by Davson, H., and Danielli, J. D. (1952). In *Permeability of Natural Membranes*, p. 83. Cambridge University Press.]
MERYMAN, H. T. (1968). *Nature, Lond.*, **218**, 333–336.
LOVELOCK, J. E. (1953). *Biochim. biophys. Acta*, **11**, 28.
LOVELOCK, J. E., and BISHOP, M. W. H. (1959). *Nature, Lond.*, **183,** 1394.
LEVITT, J. (1966). In *Cryobiology*, pp. 495–563, ed. Meryman, H. T. London & New York: Academic Press.
POLGE, C., SMITH, A. U., and PARKES, A. S. (1949). *Nature, Lond.*, **164,** 666.
RAPATZ, G., and LUYET, B. (1963). *Biodynamica*, **9,** 125.
RAPATZ, G., SULLIVAN, J. J., and LUYET, B. (1968). *Cryobiology*, **5,** 18.

POLYHEDRAL CLATHRATE HYDRATES

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Water is a very versatile substance, both at the macroscopic and at the molecular levels. It will dissolve a wide variety of substances—ionic, polar and apolar solutes. Each solute interacts with solvent to produce a unique hydrated structure which is the resultant of the interplay of the specific character of the solute molecule and the cooperative structural adaptability of the solvent molecules.

The detailed molecular structures of hydrated solute species in liquid water are difficult to establish since available probes provide only indirect information. For many solutes, however, solid crystalline hydrates can be isolated. Their molecular structures can be definitely established by X-ray diffraction techniques, and have been described for a wide variety of solutes.

In ionic hydrates the intense centrosymmetric electrostatic field orients water dipoles very sharply towards the charged centre species. On the other hand, for apolar solutes direct pair-wise interactions are very much weaker. This difference is illustrated, for example, by the extremely large hydration energies (about 100 kcal/mole [$10^3 \times 418{\cdot}4$ joules/mole]) for even singly charged ions as contrasted to values of only about 3 kcal/mole ($10^3 \times 12{\cdot}552$ J/mole) for apolar molecules such as methane or argon.

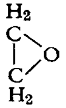
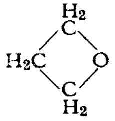
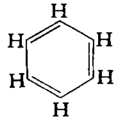
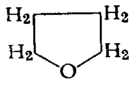
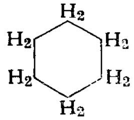
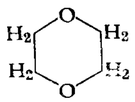
In many hydrates of apolar solutes, therefore, H_2O–H_2O interactions dominate the structure. Thus water molecules form polyhedra of very different size and shape which can enclose an apolar solute molecule as in a closed box or cage. Within recent years a very large number of different substances have been found to form hydrates with such polyhedral clathrate structures. It seems appropriate therefore to describe some of these structures as a prelude to an examination of the character of the frozen cell.

STOICHEIOMETRY AND CHEMISTRY

On the basis of chemical stoicheiometry as well as molecular structure studies it is convenient to classify the polyhedral hydrates into three major groups. These are assembled in Table I.

TABLE I

POLYHEDRAL HYDRATES

Class I		*Class II*	*Class III*
Guest Molecules			
Ar	CH_4	$CHCl_3$	$(n\text{-}C_4H_9)_4N^+F^-$
Kr	C_2H_2	CH_3CHCl_2	$(n\text{-}C_4H_9)_4N^{+-}O_2CC_6H_5$
Cl_2	C_2H_4	$(CH_3)_2O$	$[(n\text{-}C_4H_9)_4N^+]_2WO_4^=$
H_2S	C_2H_6	C_3H_8	$(i\text{-}C_5H_{11})_4N^+F^-$
PH_3	CH_3Cl	$(CH_3)_3CH$	$(n\text{-}C_4H_9)_3S^+F^-$
SO_2	CH_3SH	C_3H_7Br	$(n\text{-}C_4H_9)_4P^+Cl^-$
$C_2H_5NH_2$	CH_3CHF_2	$(CH_3)_2CO$	$(CH_3)_3N$ $(CH_2)_6N_4$
$(CH_3)_2NH$	CH_2CHF		$n\text{-}C_3H_7NH_2$ $(CH_3)_4N^+OH^-$
 		   	$i\text{-}C_3H_7NH_2$ $(C_2H_5)_2NH$ $(CH_3)_3C\text{-}NH_2$ C_4H_9OH
Stoicheiometry			
$M \cdot 5\frac{3}{4}\ H_2O$ $M \cdot 7\frac{2}{3}\ H_2O$		$M \cdot 17H_2O$ $M \cdot M'_2 \cdot 17H_2O$	$M \cdot (5\text{-}40)H_2O$
Unit Cell			
$46H_2O$		$136H_2O$	Variable number H_2O
Polyhedra		Polyhedra	Polyhedra
$2H_{12}$ (5 Å) $6H_{14}$ (6 Å)		$16H_{12}$ (5 Å) $8H_{16}$ (7 Å)	H_8, H_{12}, H_{14}, H_{15} H_{16}, H_{17}, H_{18}, H_{60}, etc.
Faces		Faces	Faces
Pentagons Hexagons		Pentagons Hexagons	Quadrilaterals Hexagons Heptagons
$M_2 \cdot M_6 \cdot 46H_2O$		$M_8 \cdot M'_{16} \cdot 136H_2O$	

(H_n symbolizes a polyhedron with n faces)

Listed at the top of Table I are representative examples of guest solute molecules which can be enclosed within the cage-like lattice formed by host water molecules. The guest molecules of Class I include inorganic as well as organic molecules, but in all cases the molecular size is relatively small. The earliest hydrates known (Davy, 1811*a*, *b*; Faraday, 1823;

Villard, 1896; de Forcrand, 1925) were those of relatively inert solute molecules, such as argon or the hydrocarbons. More recently, however, it has also been found that even molecules such as small amines or small cyclic oxides, which in principle could form disruptive hydrogen bonds directly to the water, can nevertheless be enclosed within a polyhedral cage (Glew, Mak and Rath, 1967; Jeffrey and McMullan, 1967; McMullan, Jordan and Jeffrey, 1967). Guest molecules in Class II are larger in size than those in Class I, but span the same range of chemical properties, including relatively inert hydrocarbons as well as molecules such as tetrahydrofuran or acetone, which in principle could form direct hydrogen bonds to water molecules. As we shall see shortly, guest molecules of Classes I and II are enclosed totally within a single polyhedral water lattice. In contrast the molecules in Class III are enclosed in larger and less symmetric polyhedra which can generally be visualized as being constituted by the fusion of several of the small polyhedra (McMullan and Jeffrey, 1959; Jeffrey and McMullan, 1967).

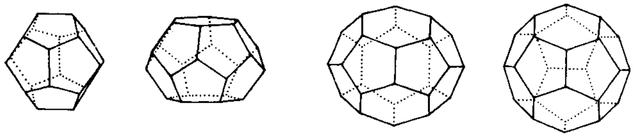
Polyhedral hydrates are also known which contain two guest species. These double hydrates are particularly common for molecules of Class II in circumstances where a second solute such as H_2S, H_2Se, CO_2, or some of the rare gases are purposely added. These have been named "helper gases" by von Stackelberg and Meinhold (1954), because their presence within the hydrate lattices very significantly increases the stability of the structure. It seems likely that, even when helper gases are not added purposely, air (i.e. oxygen and nitrogen) also participates to some extent in stabilizing the structure by being trapped within some of the open cages. This conclusion has been reached by several observers (see, e.g., Jeffrey and McMullan, 1967) who have found that gases are evolved from most hydrate crystals as they are melted.

MOLECULAR STRUCTURE

The unique characteristic feature of the structure of the polyhedral hydrates is the presence of pentagonal faces (Claussen, 1951; Pauling and Marsh, 1952; von Stackelberg and Müller, 1954). As is very well known, water is a molecule which can form tetrahedral bonds, two being contributed by the bonded hydrogen atoms and two being accepted by the two pairs of free electrons on the oxygen atom. This disposition of hydrogen atoms and free electron pairs makes H_2O particularly suited for four-coordinated interactions in condensed phases (Bernal and Fowler, 1933). Each of the two protons can form a hydrogen bond to one of the two centres of negative charge on an adjacent oxygen atom. Since each oxygen atom can act as acceptor for two hydrogen bonds and as donor for two, each water molecule is surrounded tetrahedrally by four other water molecules.

In ice this tetrahedral orientation of neighbouring water molecules extends in all directions to build up the familiar hexagonal rings of oxygen atoms in the crystal. With slight distortions in the tetrahedral bond length water molecules can also be arranged in pentagonal faces, which in the simplest situation can be fitted together to produce the pentagonal dodecahedron (Fig. 1), one of the five regular platonic solids. Alternatively polyhedra with 14, 15, or 16 faces (Fig. 1) may be produced (see Jeffrey and McMullan, 1967).

HYDRATE POLYHEDRA



	Dodecahedron	Tetrakai decahedron	Pentakai decahedron	Hexakai decahedron
Faces	12	14	15	16
Vertices	20	24	26	28
Edges	30	36	39	42
Volume enclosed	160 Å^3	230 Å^3	260 Å^3	290 Å^3

FIG. 1. Simple polyhedra found in crystalline clathrate hydrates of Class I and Class II molecules.

The dodecahedron has 20 vertices and 30 edges. At each vertex is an oxygen atom, and each edge contains an OH···O bond. Since there are 20 oxygen atoms at the vertices these must be associated with 40 hydrogen atoms ($H_{40}O_{20}$ corresponds stoicheiometrically to H_2O). Of the 40 protons, 30 can be placed in the 30 edges. Consequently ten remain to form external hydrogen bonds extending from ten vertices; and the remaining ten oxygen atoms in the dodecahedron can act as acceptors of hydrogens from external water molecules. Thus by bringing polyhedra together so that they can share faces, one can build up a superstructure which fits well into a macroscopic crystalline array. The unit cell (Claussen, 1951; Pauling and Marsh, 1952; von Stackelberg and Müller,

1954) for the Class I polyhedral hydrates (see Table I) contains eight such polyhedra, two having 12 faces and six having 14 faces. In contrast the unit cell of Class II hydrates contains, as shown in Fig. 2, 16 dodecahedra and eight hexakaidecahedra.

The dodecahedra enclose a volume of about 160 $Å^3$ and have an internal diameter of about 5·2 Å (0·52 nm) (Fig. 1). The 14-sided polyhedron has an internal diameter of 5·9 Å and a 16-sided enclosure has an internal diameter of 6·9 Å. Those guest molecules which crystallize as Class I polyhedral hydrates may fill all the cavities in a unit cell or may be inserted only in the larger enclosures. If only the larger cavities are filled by the inert molecule M then the formula for the unit cell turns out to be $6M \cdot 46H_2O$ or stoicheiometrically $M(H_2O)_{7\cdot67}$. If all the cavities are occupied by M the corresponding formulas are $8M \cdot 46H_2O$ and $M(H_2O)_{5\cdot75}$, respectively. In the Class II unit cell containing 24 cavities and 136 H_2O molecules, the guest molecules M are generally too large to occupy any of the smaller enclosures and hence fit only into the eight large cavities. Thus the formula for the unit cell is $8M \cdot 136H_2O$ or $M(H_2O)_{17}$ stoicheiometrically. It is in this group of hydrates that mixed hydrates are commonly found since a small molecule such as H_2S can fill the 16 smaller cavities. These mixed hydrates thus have the stoicheiometric formula, $M(H_2S)_2(H_2O)_{17}$.

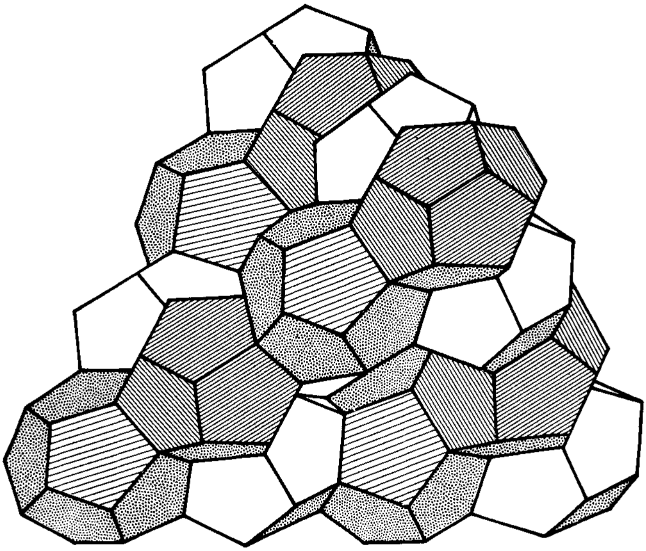


FIG. 2. Face-sharing arrangement of polyhedra in the unit cell of Class II hydrates. (See Jeffrey and McMullan, 1967.)

In both Class I and Class II hydrates each guest molecule is completely enclosed in one polyhedron with 12 to 16 faces, that is, in one of the polyhedra illustrated in Fig. 1. A skeletal diagram of the relative positions of the guest molecule in one such polyhedron is illustrated in Fig. 3 (McMullan and Jeffrey, 1965) which shows ethylene oxide in two of its possible positions within a 14-hedron. Since the interaction between the host lattice molecules and the guest molecule enclosed is a very weak one, the guest molecules have a much greater degree of rotational freedom than one would expect to find in the solid state. This is also illustrated in Fig. 4, which shows the symmetrical electron density distribution of tetrahydrofuran within the 16-hedron of its polyhedral hydrate (Mak and McMullan, 1965).

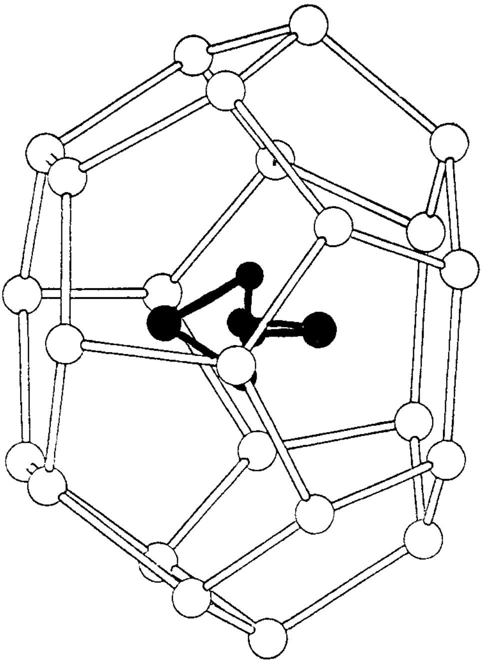


FIG. 3. Two average positions of ethylene oxide guest molecule (filled circles) within a 14-hedron (water oxygens shown as open circles). (See McMullan and Jeffrey, 1965.)

Class III polyhedra are much more complicated than those of Class I or Class II (see Fig. 5). They demonstrate that it is not necessary to squeeze a guest molecule into a simple single small polyhedron in order to have a stable hydrate. The cages in Class III may be considered as constituted from the fusion of several 12–16 hedra into a single much larger poly-

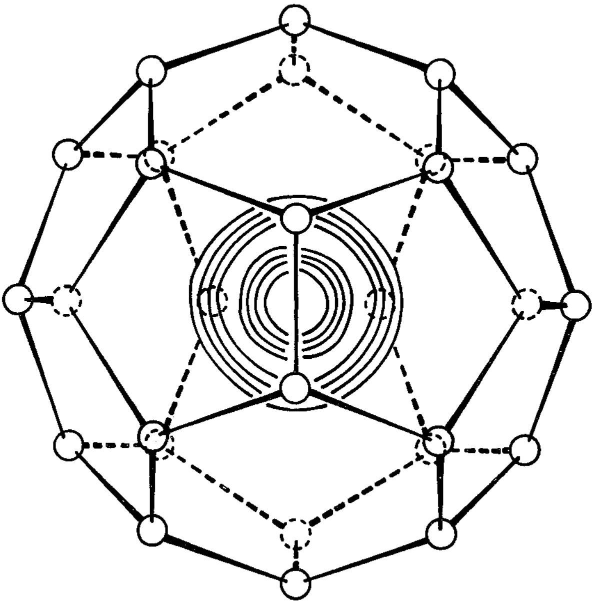
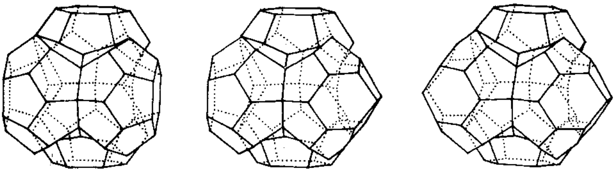


FIG. 4. Electron density distribution of tetrahydrofuran within the hexakaidecahedron of its clathrate double hydrate $8C_4H_8O \cdot 6 \cdot 4H_2S \cdot 136H_2O$. (See Mak and McMullan, 1965.)

MULTIPLE FUSED POLYHEDRA



Fusion of	4(14-hedra)	3(14-hedra) 1(15-hedron)	2(14-hedra) 2(15-hedra)
Faces	44	45	46
Vertices	70	72	74
Edges	112	115	118
Volume enclosed	1000 Å³	1000 Å³	1000 Å³

FIG. 5. Fused polyhedra found in crystalline clathrate hydrates of Class III molecules.

hedron (McMullan and Jeffrey, 1959; Jeffrey and McMullan, 1967). Some examples are shown in Fig. 5. As is evident in a comparison of Figs. 1 and 5, the fused polyhedra are composed of many more water molecules and enclose much larger volumes. Consequently guest molecules of much larger size may be contained within the cages. Fig. 6 shows the orientation of a tetraisoamylammonium ion within its polyhedral cage, which is actually constituted from two 14 hedra and two 15 hedra.

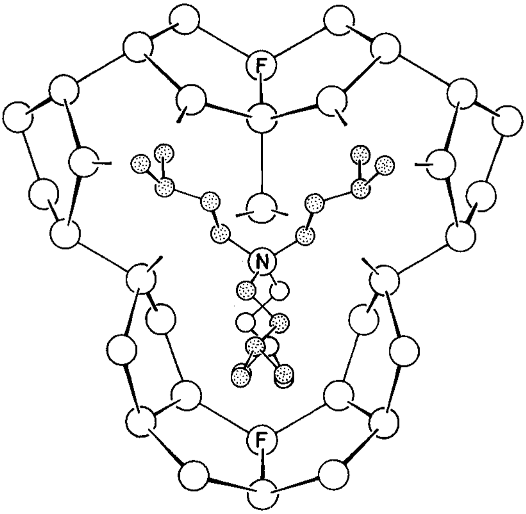
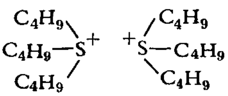


FIG. 6. Orientation of tetraisoamylammonium cation within lobes of fused (two 14- and two 15-) polyhedra of water molecules in $(i\text{-}C_5H_{11})_4N^+F^-\cdot 38H_2O$. (See McMullan and Jeffrey, 1959.)

All of the polyhedra shown so far are made up of faces containing pentagons or hexagons. Fig. 7 (Beurskens and Jeffrey, 1964) shows an even further departure from the relative symmetry illustrated so far. The cages in Fig. 7 are much more unsymmetrical and complex and are bounded by quadrilateral rhombuses as well as by pentagonal and hexagonal faces. The cavities are both large and irregular and the volume enclosed, 1600 Å^3, is substantially larger than that of any previously illustrated polyhedron. The hydrate structure shown in Fig. 7 has the formula $(n\text{-}C_4H_9)_3S^+F^-\cdot 23H_2O$. Only half of the complete water cage is shown in Fig. 7; the remaining half would be a mirror image of that illustrated. Within a full cage are two guest molecules:



$$(C_4H_9)_3S^+ \qquad {}^+S(C_4H_9)_3$$

Particularly striking is the fact that the two triplets of apolar butyl groups of the two molecules face away from each other; they tend to be far apart and embedded in (three pairs of subsidiary) water lobes rather than adjacent to each other and excluding intervening water molecules. The six incomplete polyhedra forming the lobes enclosing each of the alkyl side chains are symmetrically arranged about the centre of symmetry at the mid-point of the $S^{+}\cdots S^{+}$ line. There are three types of these incomplete compartments with different sizes and shapes made up of the following polygonal faces: (*a*) nine pentagons and two hexagons; (*b*) seven pentagons, two hexagons and one quadrilateral; and (*c*) eight pentagons and one hexagon.

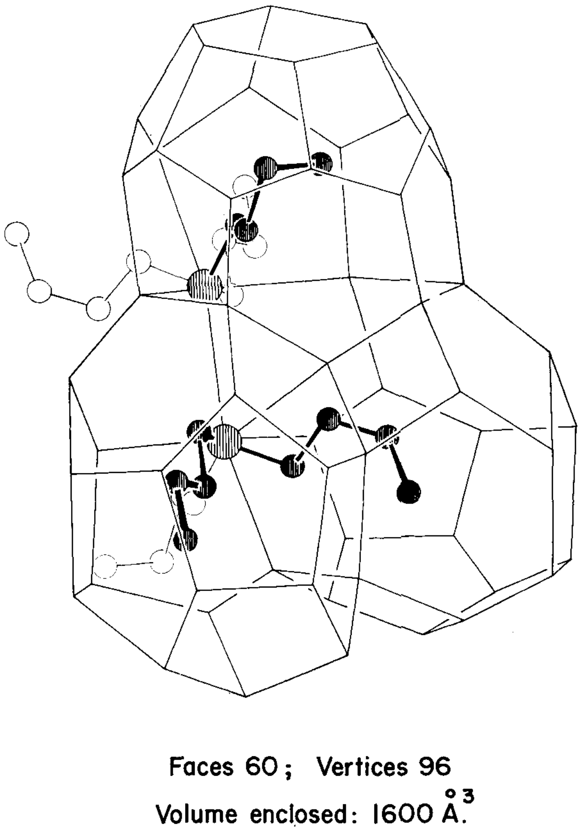
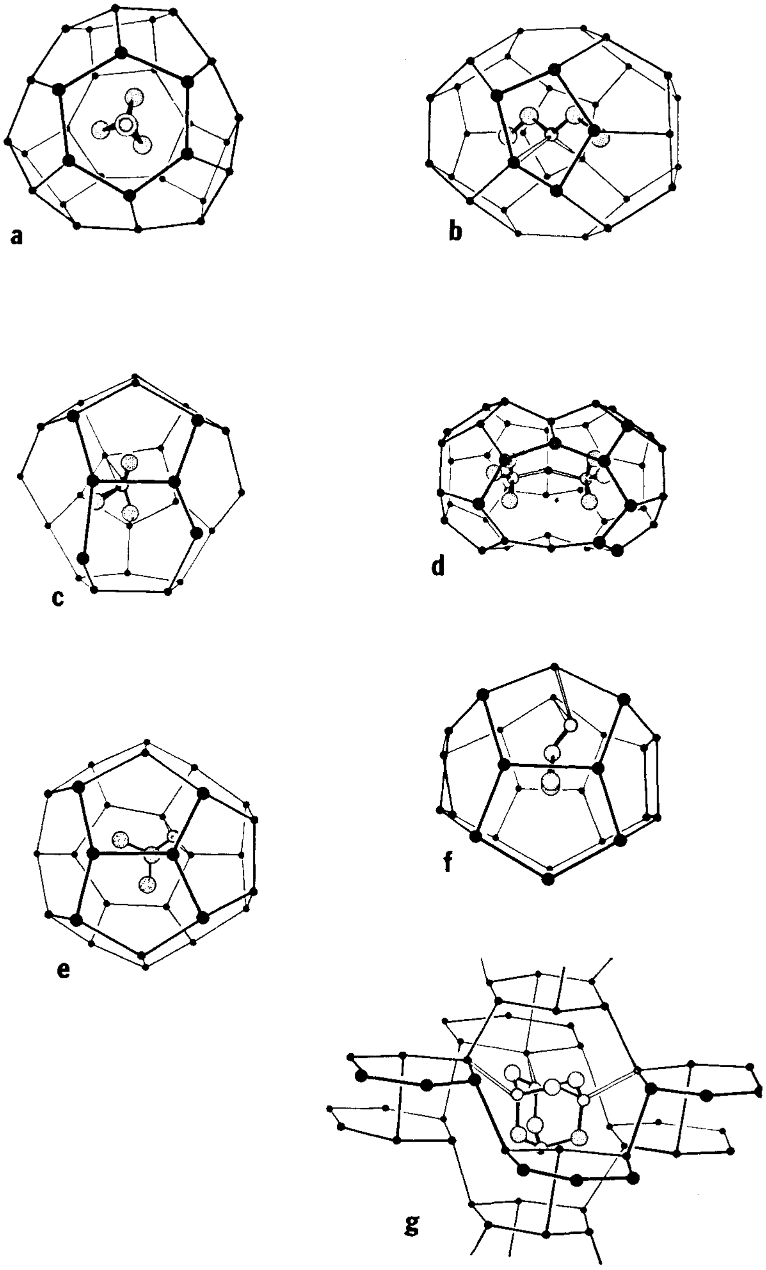


Fig. 7. Disposition of butyl side chains within lobes of polyhedra of water molecules in $(C_4H_9)_3S^+F^-\cdot 23H_2O$. (See Beurskens and Jeffrey, 1964.)


a
b
c
d
e
f
g

More recently newer types of host lattices quite different from those shown above have also been discovered (Jeffrey, 1969; Jordan and Mak, 1967; Mak, 1965; McLean and Jeffrey, 1967; Panke, 1968; Schwarzenbach, 1968). One of the most interesting of these is found in the hydrate of hexamethylenetetra-amine (Mak, 1965; Mak and Jeffrey, 1965) whose structure is shown (together with those of other amine hydrates) in Fig. 8. In this case the host lattice cannot be described in terms of a regular or even a semi-regular polyhedron. Furthermore, in contrast to the other polyhedral hydrates, in hexamethylenetetra-amine hydrate there is definite hydrogen bond formation between three of the four nitrogen atoms in the guest molecule and the water molecules in the host lattice. The interaction, therefore, between the water and the guest molecule is thus stereochemically specific.

Another interesting clathrate structure is seen in tetramethylammonium hydroxide pentahydrate (Fig. 9). The host molecules in this case are arranged at the vertices of truncated octahedra (McMullan, Mak and Jeffrey, 1966). A truncated octahedron is constituted of eight hexagonal faces and six square faces and contains 24 vertices which act as the sites of oxygen atoms of 24 water molecules. In $(CH_3)_4N^+OH^-\cdot 5H_2O$ there is a deficiency of one proton needed to form a full regular truncated octahedron. In consequence the shell is open at the bottom, as shown in Fig. 9. As a result the polyhedron can also expand, as is actually necessary in order to provide enough room for the large cationic guest molecule.

Thus it is apparent that water is a remarkably versatile substance in regard to hydrate formation, being capable of forming a large variety of cage-like structures to accommodate itself to a whole gamut of solute molecules.

FIG. 8. Structures of some amine clathrate hydrates. (See Jeffrey, 1969.) Carbon and nitrogen atoms are shaded, water oxygen atoms are black circles. Black lines are O···H···O bonds; double lines are N···H···O bonds.

(*a*) $(CH_3)_3CNH_2\cdot 9{\cdot}75H_2O$; amine non-bonded within 17-hedron.
(*b*) $(C_2H_5)_2NH\cdot 8{\cdot}66H_2O$; amine nitrogen bonded to host water molecules within 18-hedron.
(*c*) $(CH_3)_3N\cdot 4{\cdot}25H_2O$; amine nitrogen bonded to host water molecule within 15-hedron.
(*d*) $(CH_3)_3N\cdot 4{\cdot}25H_2O$; two amines bonded to additional water molecule within 26-hedron.
(*e*) $(CH_3)_2CHNH_2\cdot 10H_2O$; amine nitrogen bonded to host water molecule within 16-hedron.
(*f*) $(CH_3)_2CHNH_2\cdot 10H_2O$; amine nitrogen bonded to host water molecule within 14-hedron.
(*g*) Structure of hexamethylenetetramine hydrate, $(CH_2)_6N_4\cdot 6H_2O$.

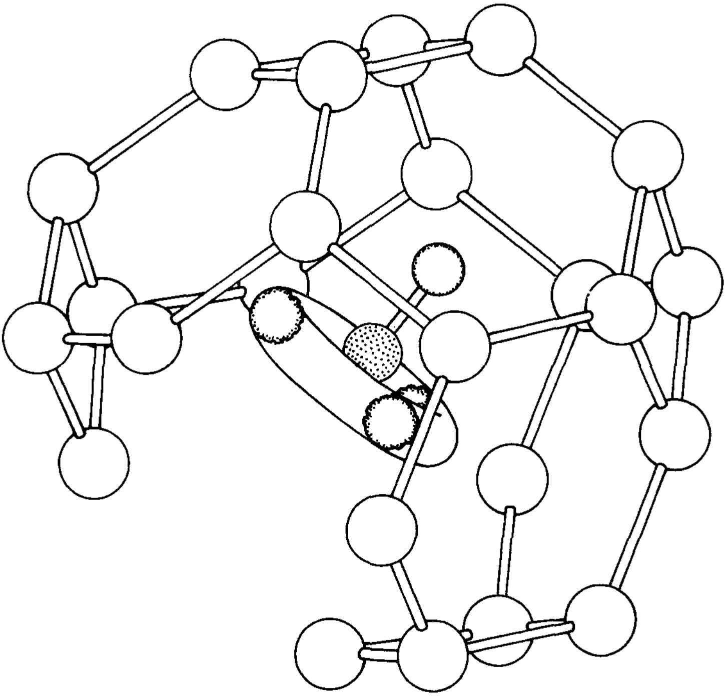


FIG. 9. Orientation of tetramethylammonium cation within cage of hydrate in $(CH_3)_4N^+OH^- \cdot 5H_2O$. Open circles are oxygen atoms of water molecules in lattice. The torus represents the rotation of the molecule about one N–C axis. (See McMullan, Mak and Jeffrey, 1966.)

SMALL MOLECULES IN SOLUTION

Given this background to the properties and structure of crystalline hydrates we might ask, what is the situation when the same solutes or corresponding ones are dissolved in liquid water? In fact, dilute aqueous solutions of non-polar solutes exhibit a number of properties which indicate stabilization of the water structure of the solvent. Table II, for example, compares the solubilization heat (ΔH°) in liquid water with the heat of formation of the clathrate hydrate for a number of non-polar solutes (Glew, 1962). For methane, for example, ΔH° when one mole of gas is dissolved in liquid water is -4621 calories ($-19\,334$ J); correspondingly the ΔH° when one mole of gas is incorporated into solid ice to form the hydrate is -4553 calories ($-19\,050$ J). It is apparent, therefore, that the energy of interaction of methane molecules in aqueous solution with the water in the hydration shell is essentially the same as