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Pharmacological actions and toxicity of dimethyl sulphoxide and other compounds which protect smooth muscle during freezing and thawing

J. FARRANT

Dimethyl sulphoxide and other non-electrolytes have been shown to protect partially, the smooth muscle of the guinea-pig uterus from damage during freezing to and thawing from −79°. The mechanism of this protection is probably physico-chemical in nature, for none of the compounds had any specific pharmacological action on smooth muscle. In the high concentrations needed for protection the compounds themselves caused some non-specific osmotic damage to the smooth muscle. All of the compounds examined had a low toxicity in mice.

IN 1949, Polge, Smith & Parkes found that glycerol protected spermatozoa from damage during freezing to and thawing from −79° and −196°. Subsequent work has shown that glycerol prevents or reduces the damage caused by freezing in a wide variety of other cells and tissues including the smooth muscle of the guinea-pig uterus (see Smith, 1961). Dimethyl sulphoxide, another water miscible non-electrolyte, was found to exert a similar action on spermatozoa and red blood cells (Lovelock & Bishop, 1959), on bone marrow cells (Ashwood-Smith, 1961 a, b) and on cells from the embryonic human lung (Porterfield & Ashwood-Smith, 1962). More recently other compounds, including pyridine *N*-oxide, methyl formamide, and methyl acetamide, have been found to protect red blood cells from haemolysis during freezing and thawing (Nash, 1961, 1962).

The ability of these compounds to protect smooth muscle during freezing and thawing has now been tested using the guinea-pig uterus. The pharmacological and toxicological properties of these compounds have also been investigated. A preliminary report of this work has been published (Farrant, 1963).

Experimental

FREEZING AND THAWING OF UTERI

In each experiment the two uterine horns from an albino guinea-pig (300–500 g) were suspended in a modified Krebs solution (NaCl 118, KCl 4·54, $CaCl_2$ 1·4, $MgCl_2$ 1·16, dextrose 11·1, $NaHCO_3$ 25, NaH_2PO_4 0·116; all in mM/litre) at 37° in two separate organ baths. A mixture of 95% O_2 and 5% CO_2 was bubbled through the solutions. Responses to standard doses of histamine (1–3 μg) were recorded using isotonic levers. The uterine horns were then transferred to separate tubes containing 10–20 ml of a solution at 37° of dimethyl sulphoxide or one of the other protective compounds. The concentration of these protective substances were, in each case, 1·4M, and the concentrations of salts and dextrose in the solutions were the same as in the original modified Krebs solution.

From the Division of Experimental Biology, National Institute for Medical Research, Mill Hill, London, N.W.7.

The temperature was maintained at 37° and 95% O_2 and 5% CO_2 bubbled through for 30 min. The uterine horns were then transferred to tubes containing 2 ml of the same modified Krebs solution containing the test substance. The tubes were placed in the cooling chamber of the freezing apparatus (Linde, Biological Freezer 3). In this apparatus cooling is brought about by the injection of liquid nitrogen into the cooling chamber containing the samples. The controlling system was modified at the National Institute for Medical Research. The rate of cooling of the sample was measured by means of a copper constantin thermocouple and recorded potentiometrically. Unless otherwise stated, samples were cooled by approximately 3°/min to the selected temperature. When this temperature had been reached the tubes containing the uteri were transferred from the cooling chamber to beakers containing the modified Krebs solution at 37° to thaw the uteri as rapidly as possible. The solution used for thawing did not contain the protective substance. After the last particle of ice had vanished from the solution, the uterine horns were resuspended in the organ baths and attached to the isotonic lever under the same conditions of tension and magnification as before. The subsequent behaviour of the muscles was observed; standard doses of histamine were added periodically and any responses recorded. Control experiments were performed in which uteri were frozen in the modified Krebs solution without a protective substance.

PHARMACOLOGICAL ACTIONS OF THE PROTECTIVE SUBSTANCES

Three smooth muscle preparations were used for pharmacological tests *in vitro*; segments of rabbit duodenum, segments of guinea-pig ileum and guinea-pig uterine horns. The rabbit duodenal segments were suspended in Tyrode solution and the guinea-pig preparations in Krebs solution. All solutions were maintained at 37° and were bubbled with O_2 (95%) + CO_2 (5%). Isotonic responses were recorded.

WEIGHING EXPERIMENTS

Uterine horns were incubated at first in the modified Krebs solution, then in the solution containing dimethyl sulphoxide, and finally on return to the control solution. During incubation the horns were weighed every 3 min after draining off the surface fluid on the side of a beaker. Changes in weight, expressed as a percentage, were compared with the mean weight observed during the initial period in the modified Krebs solution. In some experiments, one horn of a uterus was weighed while the responses of the other horn to histamine were recorded in an isolated organ bath.

TOXICITIES OF THE PROTECTIVE SUBSTANCES

The LD50 of a 4M solution of each compound in distilled water was obtained in T.O. mice (of both sexes, weighing 15–25 g). Injections were made intravenously and intraperitoneally and there were 5 mice in each group. The long-term effects of the oral administration of some of the compounds were also studied. The maximum concentration of each

substance that the mice would drink was found by preliminary experiments. The fluid intake and weight gain of groups of 5 mice given the solutions to drink were recorded for about 6 weeks. The animals were then killed and their livers removed and fixed in Bouin's fluid. Paraffin sections were stained with haematoxylin and eosin for microscopical examination.

Results

FREEZING AND THAWING OF UTERI

When guinea-pig uteri were frozen to $-30°$ or to $-79°$ in the modified Krebs solution, and then rapidly thawed and resuspended in isolated organ baths, spontaneous contractions and rhythm were never recorded and responses either to the standard or to large doses of histamine could not be obtained; the muscles appeared to be dead. On the other hand, uteri frozen to and thawed from $-30°$ or $-79°$ in the presence of dimethyl sulphoxide (1·4M) were able to contract both spontaneously and in response to doses of histamine (Fig. 1). Protection against damage did not appear

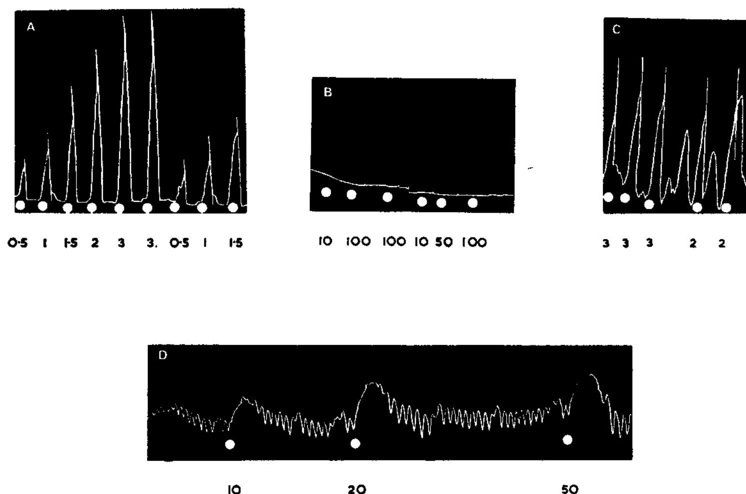

FIG. 1. Responses to histamine of guinea-pig uteri. A. Before freezing. B. After freezing to $-30°$ in the absence of DMSO. C. After freezing to $-30°$ in the presence of 1·4M dimethyl sulphoxide. D. After freezing to $-79°$ in the presence of 1·4M dimethyl sulphoxide. At the white dots histamine was added to the 15 ml bath. Doses are in μg.

to be complete. This is shown by comparing mean dose response curves to histamine from groups of uteri before freezing and after thawing from different temperatures in the presence of dimethyl sulphoxide (1·4M) (see Fig. 2). The presence of the dimethyl sulphoxide protects the muscle only partially; the lower the temperature, the greater the damage. Under these conditions most damage seems to occur between $-15°$ and $-30°$. Similar experiments were then made on uteri which had been frozen in solutions containing 1·4M of one of the other

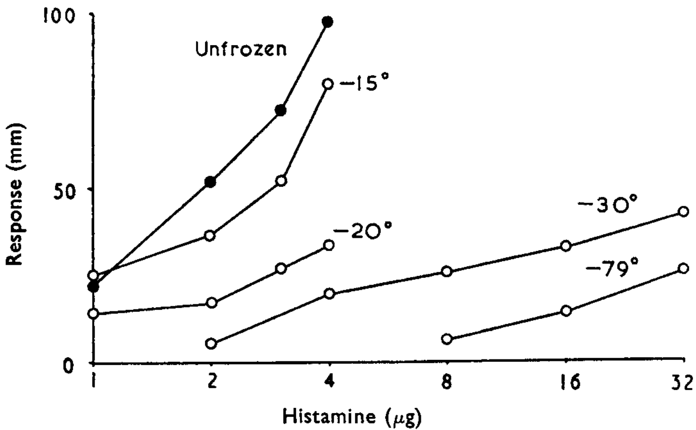

FIG. 2. Mean dose response curves to histamine from groups of uteri before freezing and after thawing from different temperatures in the presence of 1·4M dimethyl sulphoxide.

compounds. Table 1 shows that glycerol, and the four substituted amides also protected smooth muscle partially during freezing to −79°. *N*-Methylpyrrolidone and pyridine *N*-oxide did not protect the muscle.

TABLE 1. COMPOUNDS WHICH PROTECT SOME CELLS FROM FREEZING TO VERY LOW TEMPERATURES

Compound	Formulae	Protection of smooth muscle of guinea-pig uterus at −79°C
Dimethyl sulphoxide	Me_2SO	+
Glycerol	$CH_2OH(CHOH)CH_2OH$	+
Methyl formamide	HCONHMe	+
Dimethyl formamide	$HCONMe_2$	+
Methyl acetamide	MeCONMe	+
Dimethyl acetamide	$MeCONMe_2$	+
Methylpyrrolidone	$[CH_2]_3CONMe$ (ring)	0
Pyridine *N*-oxide	C_6H_5NO	0

Almost all muscles which had been partially protected from damage during freezing and thawing contracted slowly after being replaced in the Krebs solution at 37°. During the spontaneous slow contraction the addition of standard doses of histamine to the bath induced small responses which were rarely followed by a relaxation when the histamine was washed out of the bath. Furthermore, spontaneous rhythmic contractions usually occurred in the thawed preparation after the peak of the slow contraction had been reached (see Fig. 3). When spontaneous rhythm was prevented before freezing by reducing the calcium concentration from 1·4 to 0·3 mM/litre, uteri still developed spontaneous rhythm after thawing, even if the calcium was at this low level both in the freezing medium and in the thawing medium. Sensitivity to histamine increased after the spontaneous rhythm had developed, but responses to

histamine were usually less than those obtained before freezing. These points are illustrated in Fig. 3.

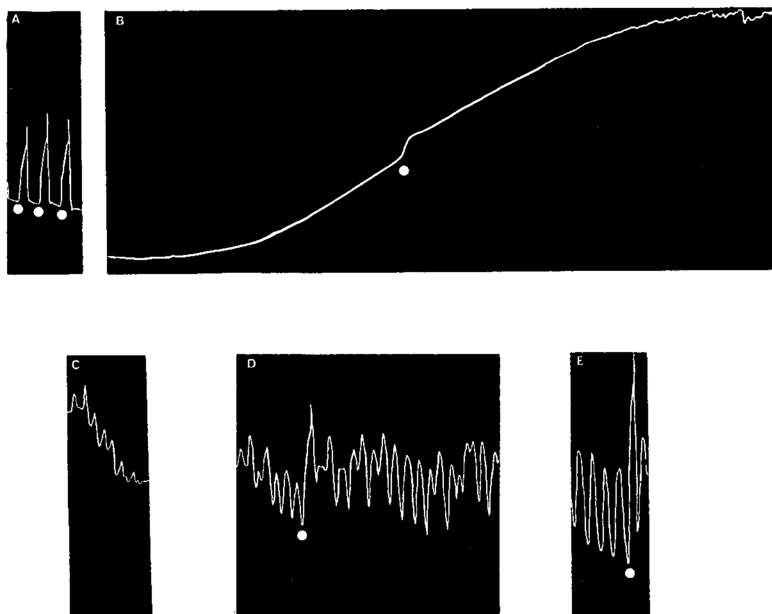

FIG. 3. Recovery of contractile ability of guinea-pig uterus after freezing to − 79° in the presence of 1·4M dimethyl sulphoxide. A. Before freezing. B. Immediately after thawing. C, D, E. 40, 60 and 120 min after thawing respectively. Histamine 0·5 μg was given at the white dots.

The effect of varying the rate of cooling was also examined. Fig. 4a records the cooling curves from two groups of experiments in which uteri were frozen in the presence of dimethyl sulphoxide (1·4M). Uteri in group A were cooled at 3°/min to −30°. Uteri in group B were cooled at 3°/min until the medium had supercooled to about −9°. Freezing then took place and the temperature rose to the freezing-point (−6°). After the freezing-point had been reached the rate of cooling was slowed because latent heat was emitted. Uteri from this group were also thawed from −30°. Fig. 4b shows that responses to histamine of muscles from group B were much less than those of muscles from group A. This result indicates that some damage occurs during freezing in the presence of dimethyl sulphoxide especially at temperatures close to the freezing point.

PHARMACOLOGICAL ACTIONS

The pharmacological properties of all the compounds listed in Table 1 have been examined on the smooth muscle of guinea-pig uterus and ileum, and on rabbit duodenum. In concentrations of up to 0·5% v/v (i.e. about 50 mM/litre) none of these compounds exerted any action alone

nor did they modify the responses to standard doses of acetylcholine, histamine or nicotine. However, when the dose was increased to 1·0% v/v all the compounds reduced the responses to acetylcholine, histamine or nicotine. These non-specific antagonisms were completely reversed after the protective substances had been washed out, for the control responses to the agonists were completely restored. Fig. 5 shows an example of this effect on the guinea-pig uterus. When the Krebs solution

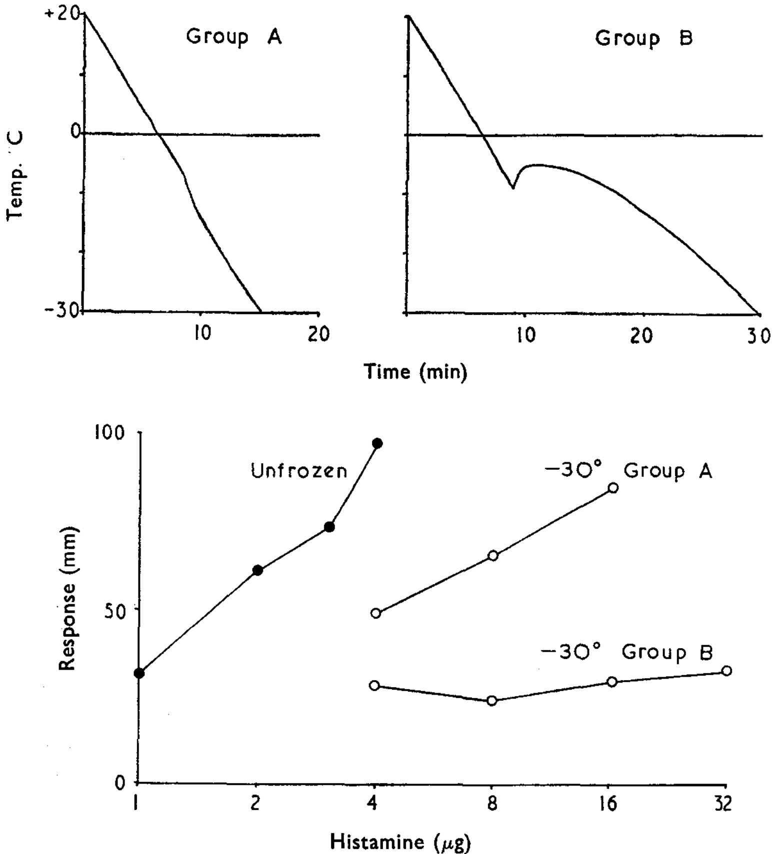

FIG. 4. a. Mean cooling curves from two groups of uteri frozen to − 30° in the presence of 1·4M dimethyl sulphoxide. b. Mean dose response curves to histamine from the two groups of uteri after thawing from − 30°.

in the bath was replaced by a Krebs solution containing 1% v/v dimethyl sulphoxide (140 mM/litre) the responses to histamine were reduced; then, when the original solution was replaced, the responses to histamine were completely restored. Fig. 5 also shows an experiment in which the uterine muscle was exposed to a solution containing 10% v/v dimethyl sulphoxide (1·4M). In this instance, the responses to histamine were completely suppressed even when large doses were given. When the dimethyl

sulphoxide was washed out of the bath there was a slow contraction of the muscle and only a partial recovery of the responses to histamine. All the protective compounds had these actions.

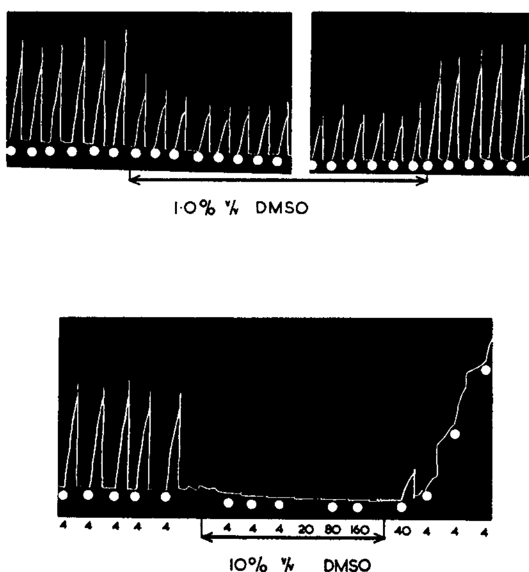

FIG. 5. Responses to histamine of guinea-pig uteri before, during and after incubation in Krebs solution containing dimethyl sulphoxide (1%, 140 mM and 10%, 1·4M). At white dots, histamine 4 μg unless otherwise marked.

WEIGHING EXPERIMENTS

Uteri incubated in Krebs solution containing dimethyl sulphoxide (10% v/v, 1·4M) lost weight but rapidly regained weight when returned to the solution without dimethyl sulphoxide. When the uteri were incubated in solutions containing lower concentrations of dimethyl sulphoxide they lost less weight (Fig. 6a). Fig. 6b records the percentage change in response to a standard dose of histamine obtained from one uterine horn after incubation in a Krebs solution containing dimethyl sulphoxide (1%, 140 mM/litre). The other horn from the same guinea-pig was treated with 1% dimethyl sulphoxide in the same way, and the changes in weight were recorded (see the top graph of Fig. 6a). When the weight of this horn had returned to normal in the presence of the dimethyl sulphoxide, the responses to histamine of the other horn were still reduced.

TOXICITIES

Acute treatment. The acute toxicity of each compound was obtained both by intravenous and by intraperitoneal injections in mice. The LD50 values in mM/kg are given in Table 2. All of the compounds examined had a very low toxicity. The least toxic substance was dimethyl sulphoxide, and the most toxic was pyridine *N*-oxide. Immediately after a

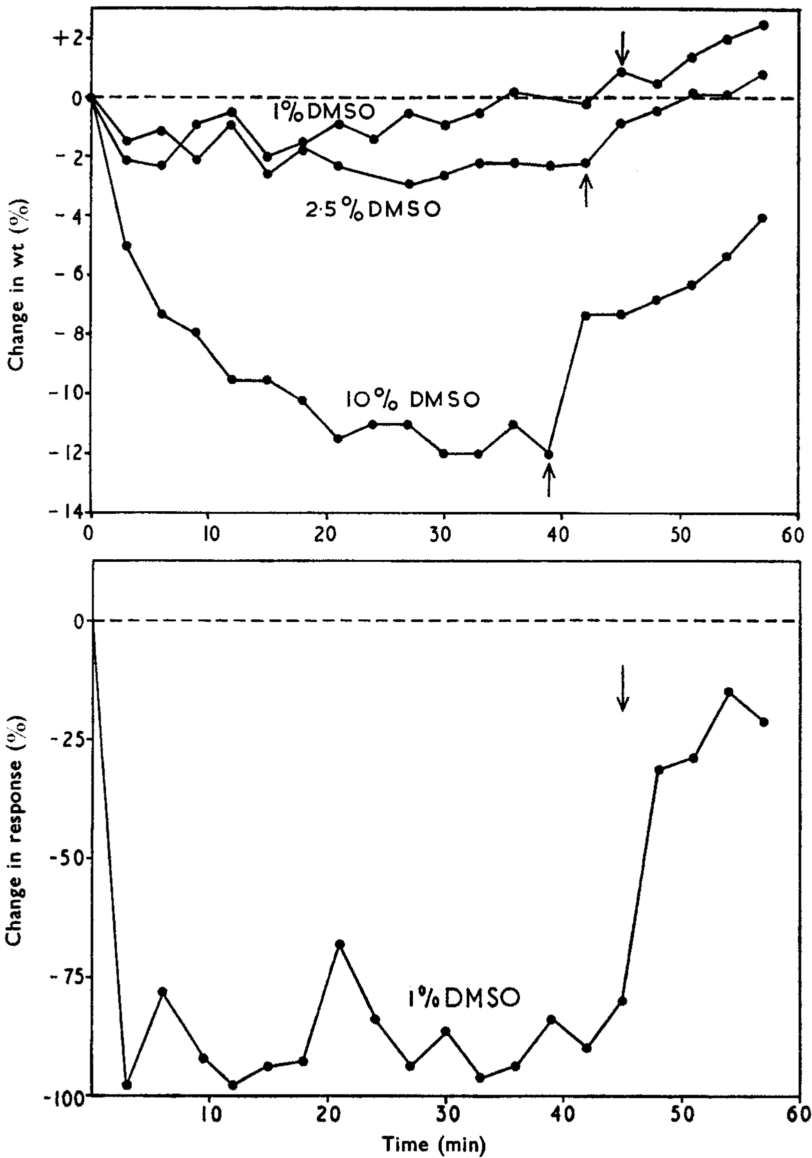

FIG. 6. a. Percentage change in weight of uteri incubated in various concentrations of dimethyl sulphoxide and after return to Krebs solution. The solutions were changed at the arrows. b. Percentage change in response to histamine of uterus incubated in 1% dimethyl sulphoxide and then returned to Krebs solution at the arrow.

lethal dose of dimethyl sulphoxide or of dimethyl acetamide the hind limbs became paralysed. A few animals died immediately after a lethal dose of a compound had been given, but most lost weight, became

hypothermic and died 2 or 3 days later. After lethal doses of glycerol intraperitoneally the animals developed ascites.

TABLE 2. TOXICITY OF COMPOUNDS IN MICE

Compound	Molecular weight	LD50 of 4M solution (mM/kg)		Maximum concentration taken orally (M)
		i.v.	i.p.	
Dimethyl sulphoxide	78	92	188	0·7
Glycerol	92	67	75	0·7
Methyl formamide	59	75	120	0·175
Dimethyl formamide	73	50	90	0·175
Methyl acetamide	73	55	60	0·175
Dimethyl acetamide	87	45	68	0·175
N-Methylpyrrolidone	99	20	36	0·35
Pyridine *N*-oxide	95	14	15	—

Chronic treatment. The maximum concentration that the groups of mice would drink is shown in Table 2. The fluid intake of mice which were drinking the dimethyl sulphoxide solution increased progressively (as shown in Fig. 7).

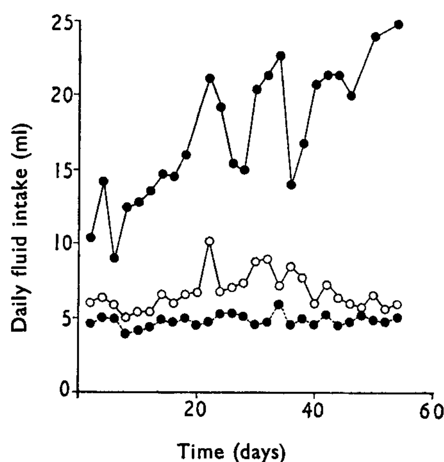

FIG. 7. Mean daily fluid intake of mice drinking dimethyl sulphoxide (0·7 M) (●—●), glycerol (0·7M) (○—○), or water (●--●).

Sections of the livers from the mice chronically treated with most of the protective agents showed mild cloudy swelling. On the other hand the livers from the mice treated with *N*-methylpyrrolidone showed severe degenerative changes. Extensive haemorrhages had taken place in some areas; in other areas fibrosis had occurred and elsewhere the parenchyma cells showed fatty degeneration.

Discussion

These results show that when the guinea-pig uterus was frozen to −30° or −79° the smooth muscle was severely damaged and no longer responded to drugs after thawing. The addition of dimethyl sulphoxide

(1·4M) to the medium partially protected the contractile mechanism of the smooth muscle during freezing so that, after thawing, responses to histamine could again be obtained. Other compounds which partially protected the muscle were glycerol, methyl formamide, dimethyl formamide, methyl acetamide and dimethyl acetamide. All these compounds are non-ionised but are sufficiently polar to form hydrogen bonds with water. Two other compounds which protected red blood cells from damage at low temperatures, pyridine *N*-oxide (Nash, 1961) and *N*-methyl pyrrolidone (Nash, private communication), did not protect smooth muscle.

Uterine smooth muscle which had been frozen in the presence of dimethyl sulphoxide was not completely protected. The lower the temperature to which the tissue was exposed the greater was the damage. Most damage occurred between $-15°$ and $-30°$. The results showed that one important factor was the length of time during which the muscle was exposed to temperatures just below the freezing point. This is indicated by the greater amount of damage observed in a group of uteri cooled slowly to $-30°$ when compared with the amount of damage in another group cooled more rapidly to the same temperature. The final temperature reached was less important. The site of damage in uteri which had been frozen in the presence of a protective substance is not clear. The fact that muscles exhibited spontaneous activity after freezing and thawing in solutions with reduced calcium suggests that the cell membrane may have been damaged.

None of the compounds tested had any specific pharmacological activity in high doses. The mechanism by which they protect the muscle from damage due to freezing and thawing must therefore be physico-chemical and not pharmacological. Lovelock (1953, 1954) suggested that glycerol and other neutral solutes entered the red blood cells and protected them during freezing and thawing by exerting their colligative properties. The same effect of lowering the concentration of salts in the solution from which ice is separating at any temperature during freezing may account for the protection of the smooth muscle. In the high concentrations required for protection they reduced or abolished responses to agonists non-specifically. These reductions in response may be due to osmotic effects, because when incubated in 1·4M dimethyl sulphoxide uterine horns rapidly lost weight. Incubation in a hypertonic solution is known to reduce the maximum tetanic tension in skeletal muscle (Howarth, 1958). Dimethyl sulphoxide may also inactivate the contractile process by some other mechanism, for, when uteri had been incubated in 140 mM/litre dimethyl sulphoxide until the weight had returned to the control value, responses to standard doses of histamine were still blocked.

All the compounds tested were remarkably non-toxic in mice. The LD50 of dimethyl sulphoxide when injected intraperitoneally as a 4M solution was 188 mM/kg. Under similar conditions the LD50 of sodium chloride is 53 mM/kg (Spector, 1956). The LD50 of dimethyl sulphoxide when injected intravenously as a 4M solution was 92 mM/kg. When

injected undiluted an LD50 of 48 mM/kg has been reported (Rosenkrautz, Hadidian, Seay & Mason, 1963). The greater toxicity of the undiluted substance may be due to the large heat of mixing. Other reported LD50 values in mice are those for dimethyl sulphoxide, 140 mM/kg intraperitoneally (Ashwood-Smith, 1961b); and for pyridine *N*-oxide, 14 mM/kg intraperitoneally (Nash, 1962). Brown, Robinson & Stevenson (1963) found that the LD50 for dimethyl sulphoxide in rats was greater than 7·5 ml/kg (about 100 mM/kg) when administered intraperitoneally.

The lack of toxicity of the compounds listed in Table 2 is confirmed by the experiments in which mice drank solutions of the compounds for a long period. Serious liver damage only occurred in those mice which had been drinking *N*-methylpyrrolidone.

At very low temperatures, cells should not deteriorate due to ageing. This is because metabolism is arrested and the physical and chemical processes involved in the degeneration of cell structures are greatly slowed. Until fairly recently these temperatures could not be used for the storage of living mammalian cells because the process of cooling to and thawing from these low temperatures caused severe damage. Although some simple organisms and particularly those which can withstand desiccation have natural systems for protection against damage during freezing and thawing, only a few types of mammalian cells could be frozen and thawed successfully without any special precautions. New possibilities were opened up by the discovery that glycerol and other compounds would protect the spermatozoa and red blood cells of several mammalian species. A wide variety of other mammalian cells and tissues have been stored at low temperatures in the presence of one or other of the protective substances (see Smith, 1961).

If a whole organ is to be stored in this way the protection of a high proportion of all the different types of cell is essential. Each cell type requires different conditions. Smooth muscle is being investigated because it is vital for the normal function of much of the digestive, excretory and reproductive systems and also it forms a major component of the vascular supply to organs. The compounds examined possess some of the ideal properties needed. They have an extremely low toxicity to the host, and therefore their removal from the stored organ before grafting may not be essential. They have no specific pharmacological effect on the tissue to be frozen.

At least three major problems remain. Firstly, in the concentration needed for protection, all the compounds so far tested themselves damaged the smooth muscle to some extent. This damage may have been due in part to osmotic changes. Secondly, protection during freezing and thawing was only partial even under the best conditions so far obtained. The damage which occurred was different from and in addition to that caused by the compound before freezing. The third problem is to determine the nature of the damage to the smooth muscle cells so that it can be reduced or prevented. Progress is already being made along these lines, but there is no immediate prospect of storing a whole organ at low temperatures.

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