Problems in the resuscitation of mammals from body temperatures below 0°C

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[Introduction]

Two hundred and seventy-five years have passed since Robert Boyle discovered that extreme cold prevented the putrefaction of animal tissues. He found that frogs and fish actually survived for short periods when the water surrounding them had frozen, but succumbed after several days’ encasement in ice. Boyle described these as promiscuous experiments. He also reported two modes of death in humans exposed to intense cold. Usually the extremities were gradually invaded by numbness which spread over the entire body so that the individual died insensibly. By contrast, horsemen wearing armour were seized violently around the waist by the cold. It caused them unspeakable abdominal pains and other torments which continued until the subjects died from exhaustion (Boyle 1683).

Boyle’s observations have since been amply confirmed, and today it is well known that cold blooded animals do not survive complete freezing of all their body water (Scholander et al. 1953). Warm blooded animals, including the hibernators, are even more sensitive to chilling. Their breathing and heart beats stop at deep body temperatures well above freezing point. The animals do not recover spontaneously when rewarmed and were therefore assumed to be dead (Adolph 1951; Lyman & Chatfield 1955). A few years ago there seemed little possibility that mammals could be resuscitated from body temperatures below 0°C, and no prospect whatsoever that the work on storing isolated mammalian cells at very low temperatures would ever be applicable to the intact animal. There were, however, reports from Russia that bats and ground squirrels had been revived from sub-zero temperatures (Kalabuchov 1934; Murigin 1937). Then we heard that Dr Andjus of the University of Belgrade had shown that rats chilled till breathing and heart beats stopped were not necessarily dead (Andjus 1951). We developed his techniques at Mill Hill so that rats and mice can now be easily revived after an hour of suspended animation at body temperatures just above zero (Andjus & Smith 1955; Andjus & Lovelock 1955; Goldzveig & Smith 1956). Meanwhile Dr Parkes and Dr Lovelock and I had found that golden hamsters survived respiratory and cardiac arrest at deep body temperatures below 0°C (Smith, Lovelock & Parkes 1954). In some animals the deep body temperature fell as low as –5°C without the formation of ice in any of the tissues. These supercooled hamsters were readily resuscitated and recovered fully. Others froze progressively until, when the internal temperature had been below freezing point for 1 h, they were rigid and, when supported only by the neck
and tail, would uphold an additional load equivalent to their own body weight of 100 g. Such animals were completely re-animated by warming the whole body with diathermy and simultaneously giving artificial respiration. The skin and superficial tissues contained large quantities of ice. Nevertheless, the extremities showed no signs of frostbite unless they had been forcibly bent when frozen (Smith 1954). The eyes, which were sometimes clouded for a short while after thawing, usually cleared completely, although occasionally lens opacities developed later. Ice crystals were also present in the brain and internal organs. Calorimetry suggested that as much as 50 % of the body water had frozen in some animals which recovered fully. This work, which was reported last year (Smith 1956a, b; Lovelock & Smith 1956) has raised many problems. Some of these problems arose when we tried to repeat the experiments on larger mammals.

Problems involved in freezing and resuscitating larger mammals

Choice of animals

Thirty-seven young adult Dutch rabbits which had been reared at Mill Hill were used. They weighed 1 to 2 kg. Rabbits were chosen because of their size in relation to hamsters, and not because of any indication that they would withstand experimental hypothermia.

Six adult prosimian primates of the species Galago crassicaudatus agisymbanus (see Osman Hill 1953) were imported from the island of Pemba near Zanzibar. They were kept for 3 to 15 months and fed chiefly on milk, mealworms, raw new born mice and bananas, with occasional hen's eggs and oranges. They would eat almost any soft food in preference to the hard pellets (Diet 31) fed to rats and mice. They weighed 700 to 1000 g at the time of cooling. Galagos were selected because they are among the smallest and the most abundant of the African primates. In contrast to Rhesus monkeys they take well to captivity and are easily tamed and handled. There seemed a possibility that they would tolerate hypothermia particularly well because some of the lemurs, to which they are related, show seasonal fluctuations of body temperature and become hypothermic under natural conditions. Rectal temperatures of 18·5° C have, for example, been recorded from Cheirogaleus major and C. medius (Bourlière & Petter-Rousseau 1953). Deep body temperatures below 36° C were not, however, recorded from the galagos while they were living in the animal house at Mill Hill.

Methods of cooling

In preliminary experiments hypothermia was induced by anaesthetizing rabbits with nembutal (approximately 0·5 g per kg body weight intraperitoneally) and immediately immersing them in ice-cold water. These animals were used to test the rates of cooling and rewarming. In later experiments, when the animals were to be resuscitated, hypothermia was initiated by a modification of the closed vessel technique previously used for rats and hamsters (Andjus & Smith 1955; Smith 1956a). The rabbit was enclosed in a Perspex box, 10 l. in capacity, in a cold room at −2° C. Two to four litres of carbon dioxide was passed through the box until the animal became anaesthetized. One litre of oxygen was introduced after 5 to
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10 min and subsequently at intervals of approximately 30 min. After 3 to 4 h the animals showed no spontaneous movements. They were breathing about forty times per minute. They were removed from the box and immersed in icy water containing crushed ice.

The deep body temperature was recorded from thermocouples in the descending colon, the peritoneal cavity, and the thoracic part of the oesophagus. Subcutaneous and intramuscular temperatures were also recorded. Breathing stopped at deep body temperatures between 25 and 30°C in the animals anaesthetized with nembutal, and between 13 and 21°C in those treated with carbon dioxide. The heart stopped beating 1 to 2 min later when the temperature had dropped by another 3 or 4°C. The animals were then immersed in baths at −5°C. Thereafter the deep body temperature fell less rapidly than in the hamsters, and took 1 h to pass from 15 to about 10°C. By this time the extremities had been freezing for 20 to 40 min. Methods of increasing the rate of cooling were therefore investigated. No attempt was made to cool the blood stream because the heart had stopped beating. Instead, the stomach and the rectum were cooled internally by means of an ice cream mixture. This mixture consisted of goat serum diluted 1 in 4 with normal saline, frozen at −2°C, and stirred vigorously in a high-speed mechanical blender until the ice crystals were finely divided and the mixture creamy. 20 to 50 ml. quantities were injected through tubes inserted into the stomach and rectum. The thawed fluid was withdrawn after 1 min and replaced by more ice cream. As a result the deep body temperature fell rapidly, passing from 15 to 0°C within approximately 40 min. The method was abandoned because the gastric mucous membrane was extensively damaged, and in several instances the stomach was ruptured. We therefore concentrated on increasing the rate of heat loss from the surface of the body during immersion in the bath at −5°C. After the fur had been shaved from the entire trunk the deep body temperature fell from 15 to 0°C within 45 min. This method, which was not applicable to rabbits which were to be resuscitated, suggested that the insulating properties of the fur must be abolished. The technique finally adopted was to wash the rabbit in icy water containing a synthetic detergent until the undercoat was thoroughly wetted and until no air was enmeshed in the fur. The abdomen was shaved. The animal was then transferred to a large bath of propylene glycol kept at −5 to −6°C and stirred vigorously by jets of compressed air. By this means the deep body temperature was reduced to the freezing point of plasma within 1 h of cessation of breathing and 20 to 40 min after the extremities had begun to freeze. The twelve rabbits used in these preliminary experiments were used to test the rate of rewarming by diathermy.

Four of the galagos were cooled by the method used for rabbits. The other two were enclosed in the 10 l. Perspex box and left at −2°C without addition of carbon dioxide or oxygen. They reacted in the same way as rats and hamsters cooled in closed vessels and gradually became hypothermic and comatose during the next 2½ to 3 h. At the end of that time they were transferred to icy water until breathing stopped at temperatures between +7 and +9°C. They were then immersed in vigorously stirred baths of propylene glycol at −5°C to reduce the deep body temperature below 0°C and to freeze the tissue fluids.
Methods of rewarming

By the time the internal organs had begun to freeze the extremities and superficial tissues had been freezing for about 40 min. The quantity of ice formed and the energy input needed to thaw them was, therefore, much greater than in the hamsters. No suitable source of energy was available until Dr Lovelock built a powerful diathermy apparatus which he has described (see p. 545, Lovelock 1957). At first, currents conducted round the surface caused severe superficial burns before the interior of the rabbits had thawed. When this hazard had been overcome the entire trunk became thawed and the viscera became cooked within a few minutes. In subsequent experiments a procedure was worked out whereby frozen rabbits were thawed within a few seconds and the deep body temperature raised from \(-0.6\) to \(+10\) or \(15^\circ\) C at the end of 1 min. The energy input was then reduced so that the temperature in the colon rose by \(1\) to \(2^\circ\) C/min. Meanwhile, cold air was blown over the surface of the animal to prevent overheating the skin or premature dilatation of its blood vessels. Figure 60, plate 25, shows a galago being given artificial respiration during rewarming in the diathermy apparatus. Its pupils are widely dilated. It was thawed but still inanimate at the time the picture was taken.

Results of rewarming frozen rabbits and galagos

Fifteen rabbits and two galagos were treated in this way. In each instance the heart which had, of course, been at a standstill, resumed beating when the internal temperature reached about \(15^\circ\) C. The mucous membranes and skin became pink. The pupils constricted and the animals breathed spontaneously at colonic temperatures varying from \(20\) to \(30^\circ\) C. Diathermy and artificial respiration were then discontinued and the surface of the body was gently warmed. The galagos and some of the rabbits were left under a radiant heat lamp. Other rabbits were either put in a warm incubator or immersed in warm water. A few were left at room temperature. Muscle tone improved and the animals made spontaneous movements. Some of them, including the two galagos, sat up and moved around. Within about an hour, however, the reanimated rabbits and galagos all collapsed and died. At post mortem the only obvious lesion was a severe haemorrhage in the upper part of the stomach. This is the part of the stomach which secretes hydrochloric acid. The normal histology of the fundus of the rabbit stomach is shown in figure 61, plate 25. The large cells with pale nuclei and much cytoplasm are the oxyntic cells which are responsible for secreting hydrochloric acid. The cells with darkly stained nuclei and comparatively little cytoplasm are the peptic cells. The cells which form the inner lining of the mucosa are the mucus secreting cells. Microscopic studies of stomachs from the frozen and partially resuscitated animals showed that bleeding was restricted to regions in which oxyntic cells were present. Figure 62, plate 25 shows part of the haemorrhagic area from one of these stomachs. The remnants of gland tubules lined by peptic and by oxyntic cells are separated by red blood cells and fibrin.

The pyloric part of the stomach, where there are no acid secreting cells, was normal. Dr Lovelock and I wondered, therefore, whether hydrochloric acid was
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the cause of damage. Twenty years ago an identical haemorrhagic lesion in rabbit stomachs was described and illustrated by Sir Charles Dodds and his co-workers (Dodds, Noble, Scarff & Williams 1937). They produced the damage by administering posterior pituitary extracts to animals with normal body temperatures and showed that the acid secretion of the stomach played an important part in producing the damage during the period of vascular constriction in the stomach wall. Monkeys, cats, guinea-pigs and rats developed similar lesions in response to posterior pituitary extract. I had noticed gastric haemorrhage in hamsters which had been frozen and which were killed or had died shortly after resuscitation (Smith 1956b). The haemorrhage was in the lower half of the hamster stomach (see figure 64, plate 26. I had previously thought that this was the pyloric part of the stomach where there should be no acid secreting cells. Then Professor Amoroso told me that the upper half of the hamster stomach is analogous to a rumen. Histological studies showed that it is lined by stratified squamous epithelium, which is continuous with the mucosa of the oesophagus, and which may spread over a small area of the lower compartment of the stomach. The haemorrhagic part of the stomach in the frozen hamsters is the region which secretes acid. Its histological appearance is similar to the rabbit stomach shown in figure 62, plate 25.

At normal body temperatures the mucus-secreting cells which form the inner lining of the stomach and the cells which line the glands actively exclude hydrochloric acid. When body temperature falls and after circulatory arrest this selective impermeability might well be lost because of the reduced metabolic rate of the cells. By comparison with biochemical processes diffusion is slowed relatively little at reduced temperatures. In hypothermic animals hydrochloric acid already present within the lumen of the stomach could, therefore, diffuse throughout its wall injuring the component cells including those forming the walls of blood vessels. When the animals were rewarmed and when circulation was restored the damaged blood vessels would bleed.

We tested this theory by neutralizing the contents of the stomach with sodium bicarbonate before breathing and heart beats stopped, and before the hypothermic rabbits were immersed in the freezing bath. The animals were then frozen in the usual way until the deep body temperature was at the freezing point and until the extremities had been freezing progressively for 45 min. They were resuscitated and 2 h later were killed to examine the stomach. There was no sign of gastric haemorrhage and histological sections showed that the mucous membrane of the fundus of the stomach was indistinguishable from normal (see figure 63, plate 25). Dodds and his co-workers had previously shown that neutralizing the gastric contents prevented the lesions which otherwise occurred after injection of posterior pituitary extracts (Cutting, Dodds, Noble & Williams 1937). I am most grateful to Sir Charles Dodds for drawing my attention to their work. It strengthened our opinion that the gastric haemorrhage in our animals did not depend on the formation of ice crystals in the stomach wall or on shock after thawing, but resulted from diffusion of hydrochloric acid into the mucosa when the circulation had been arrested by cold.

Unfortunately, survival was not greatly prolonged by the bicarbonate treatment. Three frozen rabbits which had been treated in this way all died within 4 h of
resuscitation. Two of the galagos regurgitated and inhaled bicarbonate from the stomach during administration of artificial respiration. The other two galagos which had been treated with bicarbonate and then frozen for 45 min seemed to make an excellent recovery after thawing. One of them regained an appetite as well as normal posture and behaviour. Within 24 h they both died. At post mortem the stomach was normal, but in one animal the duodenum and jejunum contained bloodstained fluid. In both instances there was oedema of the lungs and froth in the trachea. This may have been a terminal event. Survival may have been limited by some other physico-chemical or physiological derangement which, if diagnosed, might well have been susceptible to treatment. It was therefore decided to postpone further experiments on freezing the larger mammals until the effects on other organs of freezing in vivo and in vitro were better understood.

Studies on the placenta and other organs in frozen hamsters

Recent studies on frozen hamsters suggest that vascular stasis may persist in certain organs for some hours after resuscitation. For instance, the spleen is often enlarged and the liver and kidneys may be congested so that, when sectioned, blood oozes from them. Small haemorrhages or discoloured areas are sometimes seen on the surface of the lungs. A particularly interesting example of vascular congestion was found in the placenta. Pregnancy lasts for 16 days in the hamster. Hamsters which had been frozen for 30 min, with body temperatures below 0° C during the first 8 days or on the 12th day after fertilization of the egg, recovered completely and normal embryonic and foetal development was resumed. In due course, normal young were born, or, if killed before delivery, normal foetuses were found in the uterus. By contrast, animals frozen on the 9th, 10th and 11th days did not give birth to young, and in others killed on the 13th day of gestation the majority of foetuses were undergoing resorption and were surrounded by dark altered blood (Smith 1957). I showed these results to Professor Amoroso who immediately pointed out that the hamster placenta undergoes rapid growth and a radical change in structure between the 9th and 11th days and might well be liable to damage during this period. He very kindly undertook to collaborate in investigating the matter. Figure 64, plate 26, shows the uterus of a hamster which had been frozen on the 10th day of pregnancy. It was resuscitated and killed 3 h later. Each swelling indicates the position of a developing foetus. Several of these swellings show

Description of Plate 25

Figure 60. A frozen galago is being rewarmed with diathermy. It lies inside a Perspex tube surrounded by the output coil of the diathermy apparatus. Artificial respiration is being given by insufflating air into a tracheal cannula.

Figure 61. Section through the fundus of the stomach of a normal rabbit. (Magn. × 112.)

Figure 62. Section through the fundus of the stomach of a rabbit which had been frozen for 40 min and resuscitated, but died 2 h later. (Magn. × 112.)

Figure 63. Section through the fundus of the stomach of a rabbit which had been treated by introducing 10 ml. of 4% NaHCO₃ into the stomach before freezing. It was frozen for 40 min, resuscitated and killed 2 h later. (Magn. × 112.)
patches of dark purple discolouration due to intra-uterine bleeding. Histological sections of the individual conceptuses showed that the maternal blood sinuses in the developing chorio-allantoic placenta were engorged, whereas the foetal blood vessels in the trophoblastic tissue were not conspicuous. Some of the maternal blood sinuses had ruptured and were oozing blood. In places the trophoblastic villi were separated from the main part of the placenta by blood cells and fibrin (see figure 65, plate 26). Structure was a little confused in areas where there was much haemorrhage, but the free blood cells were invariably non-nucleated and therefore of maternal origin.

The normal appearance of a placenta at the same stage of development is shown for comparison in figure 66, plate 26. The maternal blood sinuses contain comparatively little blood. Our studies on the placenta convinced me that hydrochloric acid was not the only cause of bleeding in hypothermic or frozen mammals and that circulatory disorders might be of great importance. I wondered whether the coronary circulation of the heart might be deranged after prolonged periods of freezing, and to what degree the cardiac muscle fibres themselves would survive freezing.

**Studies on the isolated heart**

Hearts removed from hamsters at normal body temperatures continue to beat _in vitro_ for at least 8 h when the coronary circulation is perfused with a suitable nutrient fluid at room temperature. Hearts removed from hamsters which had been freezing for 30 min showed no impairment of function when perfused in the same way. Figure 52 shows the mechanical beat recorded on a smoked drum soon after thawing and 4 h later. The corresponding electrocardiograms are shown below the kymograph tracings. These records are normal, as might be expected, because intact hamsters recovered completely after freezing for 30 min. On the other hand, it was not possible to resuscitate hamsters which had been freezing for 3 h, and the heart did not resume beating in the intact animal. Nevertheless, when the hearts were removed from hamsters which had frozen for 3 h they resumed beating when perfused _in vitro_ as shown in figure 53. The beat was moderately forceful and was sustained for several hours provided that the intra-thoracic temperature had not fallen below —2° C.

**Description of Plate 26**

**Figure 64.** The stomach and uterus of a hamster which had been frozen for 30 min on the 10th day of pregnancy, 2 h after resuscitation. The dark patches on the lower compartment of the stomach and on the uterine swellings correspond to haemorrhagic areas inside the organs. Scale in cm. (Magn. × 1·5.)

**Figure 65.** Section through the chorio-allantoic placenta of a hamster which had been frozen for 30 min on the 11th day of pregnancy. Killed 3 h after resuscitation. The maternal sinuses are engorged with blood. Reichert's membrane has been disrupted and free blood has caused separation of the trophoblastic villi. (Magn. × 112.)

**Figure 66.** Section through the chorio-allantoic placenta of a normal hamster on the 11th day of pregnancy. A small amount of foetal tissue is included. (Magn. × 112.)
When the intra-thoracic temperature of the intact animal had reached a level between \(-2\) and \(-3^\circ\) C at the end of 3 h freezing, contractions of the isolated heart were irregular and could sometimes only be elicited by mechanical stimulation. This state of affairs is illustrated by the kymograph tracings shown in figure 54.

![Kymograph tracings and electrocardiograms](image)

**Figure 52.** Kymograph tracings and electrocardiograms recorded from the heart of a hamster which had been freezing progressively for 30 min until the deep body temperature reached \(-0.7^\circ\) C. (a) immediately after thawing, (b) 4 h later. Drum speeds 8 and 128 mm/min.

![Kymograph tracings and electrocardiograms](image)

**Figure 53.** Kymograph tracings and electrocardiograms recorded from the heart of a hamster which had been freezing progressively for 3 h until the intrathoracic temperature reached \(-1.5^\circ\) C. (a) immediately after thawing, (b) 1 h later. Drum speeds 8 and 128 mm/min.

Immediately after thawing from \(-2.2^\circ\) C this heart beat erratically for a few moments and then stopped until pinched. An hour later it still responded to stimulation. If the intra-thoracic temperature in the frozen hamster was between \(-3\) and \(-5^\circ\) C the auricles of the isolated heart resumed beating, but not the ventricles. Freezing to temperatures below \(-5^\circ\) C abolished all activity as judged
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by electrocardiograms and lack of visible contractions. Similarly, hamster hearts removed from warm animals and frozen in vitro recovered after freezing for short periods at \(-2^\circ\) C but not at \(-5^\circ\) C.

It was very interesting to find that the tortoise heart was more resistant than the hamster heart and would survive freezing for long periods at \(-2^\circ\) C and for short

Figure 54. Kymograph tracings and electrocardiograms recorded from the heart of a hamster which had been freezing progressively for 3 h until the intrathoracic temperature reached \(-2.2^\circ\) C. (a) immediately after thawing, (b) 1 h later. Drum speed 128 mm/min.

Figure 55. Kymograph tracings recorded from the hearts of two tortoises. (a) (1) before freezing, (b) (1) after freezing for 24 h at \(-2^\circ\) C, (a) (2) before freezing, (b) (2) after freezing for 1 h at \(-10^\circ\) C. Drum speeds 8 and 128 mm/min.
periods at $-10^\circ$ C (figures 55 and 56). The rat heart stops beating in vitro at a temperature between $+10$ and $+15^\circ$ C, whereas hamster and tortoise hearts do not stop until the temperature is almost zero. Nevertheless, the rat heart recovered completely after freezing at $-2^\circ$ C for about 1 h, and showed slight recovery after freezing to $-4\cdot8^\circ$ C (see figure 57). There was no recovery from temperatures below $-5^\circ$ C. The rat and hamster hearts were, in fact, almost identical in their resistance to freezing, although they respond so differently to cooling above zero.

These studies suggested that the heart was not the first organ to fail when intact mammals were frozen. They also suggested that the mammalian heart might with-
stand freezing to even lower temperatures if it could be permeated with glycerol. The hamster heart proved very sensitive to sudden increases in the glycerol concentration of the fluid perfusing its coronary vessels. At slightly reduced temperatures (10 to 15°C), however, glycerol could be introduced gradually without arresting the beat, until at the end of about 1 1/2 h the heart was being perfused with

![Figure 58](image)

**Figure 58.** Kymograph tracings from the heart of a hamster during cooling to +13°C and during addition of glycerol to the perfusion fluid at that temperature. (a) during cooling from +20 to +15°C before addition of glycerol, (b) addition of glycerol is begun at 13°C at point marked by arrow, (c) perfusion fluid contains 7.5% glycerol, (d) perfusion fluid contains 10% glycerol, (e) perfusion fluid contains 15% glycerol. Drum speeds 8 and 128 mm/min.

![Figure 59](image)

**Figure 59.** Kymograph tracings from the heart of a hamster which had been perfused with 15% glycerol and thawed from -20°C. The glycerol was gradually removed during perfusion at +20°C. (a) immediately after thawing, (b) glycerol concentration has been reduced to 7.5%, (c) and (d) perfusion with glycerol stopped, (e) 4 h after thawing.
15 or 20% glycerol and was still beating gently (see figure 58). Then, if the glycerol was gradually removed over the course of about 2 h the isolated heart contracted rhythmically with increasing vigour until the tracings were of the original amplitude. When frozen to and thawed from −20°C a number of glycerol-treated hearts resumed beating and, when the glycerol concentration was reduced, the force of the beat increased. Sometimes they contracted and relaxed erratically or only in response to pinching, but usually a regular beat was established as shown in figure 59. So far none of the glycerol-treated hearts has survived freezing to −79°C, but the investigation is still in its early stages and difficulties such as formation of air emboli in the coronary vessels, and failure of the heart to relax when thawed may eventually be overcome by suitable modifications of medium and of technique.

This is an opportunity to express my warm thanks to Dr A. S. Parkes, F.R.S., who encouraged these researches and to my colleague Dr J. E. Lovelock who built the diathermy apparatus and advised and helped in some of the experiments. I am much indebted to Professor E. C. Amoroso, F.R.S. of the Royal Veterinary College for his collaboration and for the photograph, taken by Mr A. R. Goffin, which is reproduced in figure 64, plate 26. Figure 60, plate 25, was taken by Mr C. Sutton, and the photomicrographs in plates 25 and 26 were the work of Mr M. Young of the National Institute for Medical Research. Miss S. Gibson helped in the experiments. Mr Oliver Jones, M.R.C.V.S., of the Zoological Society of London, kindly advised about the care of galagos.

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