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Perfusion Preservation of Hearts for 6 to 9 Days at Room Temperature

Abstract. *The combination of a defined medium with single-pass perfusion has made possible long-term maintenance of beating rat hearts at 22°C in vitro. The 6- to 9-day survival period appears to be the longest so far reported for hearts. This method provides a stable system which should be useful for investigating the role of single factors in myocardial preservation and evaluating the effects of exposure to pharmacological and toxicological agents.*

Techniques for maintaining single cells or small tissue sections in culture for extensive periods have been known for several decades. However, the maintenance in vitro of perfused parenchymatous organs, such as the heart and kidney, has been severely limited. The development of transplantation medicine has focused renewed attention on perfusion techniques for the maintenance of organs in vitro, and it is essential to preserve cadaver organs for sufficiently long periods to permit accurate cross matching and transportation [for reviews, see (*1*–*5*)]. The diversity of the approaches to preservation has precluded a general consensus on the factors that limit survival (*5*, *6*). Attempts have included a broad temperature range, various physical techniques, and different perfusion fluids. Among these, hypothermic perfusion with plasma-based fluids and cold storage after flushing with an "intracellular" (high potassium and magnesium) electrolyte solution appears clinically useful. Most current preservation techniques utilize deep hypothermia (< 10°C). The experimental preservation of hearts for 4 days after continuous perfusion with a modified Ringer's solution (*7*) and the preservation of kidneys for 7 days after perfusion with a modified plasma preparation (*8*) have been reported, but the results with kidneys were not consistently achieved, possibly because of variations in the plasma-based media. Methods involving recirculating perfusion and simple cold storage are invariably complicated by multiple, unquantifiable variables arising from tissue metabolism and cellular solute exchange even in deep hypothermia. No system has been developed in which single variables can be evaluated.

Perfusion methods are also used to maintain organs in vitro for basic physiological, biochemical, and pharmacological studies at normothermia or moderate hypothermia. Studies at normothermia or moderate hypothermia have been limited because signs of deterioration appear within 3 to 5 hours, depending whether a working or Langendorff preparation is used (*9*). Furthermore, no wholly satisfactory perfusate has been found for preserving the myocardium during open-heart surgery. There is no evidence that it is possible to maintain functioning organs in vitro for prolonged periods under normothermic or moderately hypothermic conditions.

We report here the maintenance of beating rat hearts at 22°C for 6 to 9 days (Table 1), using a defined medium (Table 2) in a single-pass perfusion system. To our knowledge, survival of functioning hearts for this length of time has not been previously reported. By using the single-pass method, the composition of the medium can be nearly completely controlled throughout the preservation period. The medium is patterned for the most part after normal plasma so that it would potentially be able to meet metabolic needs over a broad temperature range. It is not a minimum essential medium, as some components were included simply because they are present in plasma, as long as no harmful effect appeared when they were added to a modified Ringer's solution. Other components (such as putrescine) were used because they are essential for tissue culture fluids (*10*) or (such as adenosine) for physiological regulatory functions (*11*).

Table 1. Survival times of hearts perfused with synthetic plasma-simulating solution at 22°C. The criteria for survival are described in the text. All gas mixtures included 5 percent CO_2 to maintain the *p*H at 7.4. The numbers of hearts are given in parentheses.

Gas mixture (% O_2, % N_2)	Survival times (days)
0.0, 95.0	5 (2), 6 (1)
47.5, 47.5	6 (6), 7 (1)
63.3, 31.7	6 (3), 7 (2), 8 (2), 9 (1)

The medium was prepared in 9-liter batches by combining the stock solution and dry chemicals (Table 2) with ultrapure water while bubbling rapidly with a mixture of 95 percent N_2 and 5 percent CO_2. Water was purified in a Millipore Super Q system and, in order to eliminate any organic impurities, repurified in an Ultrascience still (model 102S). Perfusate was sterilized by pressure filtration through Millipore filters (0.22-μm pore size) and stored under the N_2-CO_2 gas mixture for a maximum of 3 days before use. Serum albumin was added to minimize the loss of insulin and glucagon by adsorption during filtration. Care was taken to avoid surface foaming after the protein components were added. The filtration through 0.22-μm filters was repeated before the medium was passed into the perfusion apparatus. The perfusate came in contact only with borosilicate glass, silicone rubber, polypropylene, and cellulose acetate surfaces.

At the start of each experiment hearts were quickly removed from decapitated Wistar rats, cannulated for coronary perfusion through the aorta (Langendorff technique), and mounted in controlled-temperature chambers. The ischemic time of hearts before the start of perfusion was limited to 2 to 3 minutes. A perfusion flow of 2 to 4 ml g^{-1} min^{-1} was maintained by having a 60- to 70-cm column of the medium in narrow-bore tubing open to the atmosphere and fed by bilateral cam action of a peristaltic pump (Harvard Apparatus, model 1203). Temperature was held constant by using refrigerated circulating temperature controllers. All procedures were carried out in a sterile area in a laminar-flow hood.

Perfusion with the synthetic medium was continued until the first signs of deterioration, such as arrhythmia and discoloration, appeared. Survival time (Table 1) was counted as the time from the start of perfusion to the time at which readily visible achromatic areas appeared on the surface of the myocardium, usually on the right ventricle near the insertion point in the pulmonary artery. Most experiments were terminated at this time. Discoloration was usually accompanied by arrhythmia. If perfusion was continued, the achromatic area spread gradually over the total myocardium. Total discoloration accompanied the eventual cessation of all signs

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of contractility. Contractions were noted as much as 15 days after the start of perfusion. Significantly, the nearly complete discoloration at 15 days was not accompanied by increased coronary resistance as determined by the perfusion rate. Discoloration appearing earlier than 4 days was usually randomly distributed and was accompanied by increased coronary resistance, which could be ascribed to particle or air embolism or to bacterial contamination.

When perfusion was terminated at the first signs of achromatic areas, hearts were routinely sectioned, blotted on Whatman No. 1 filter paper wetted with the medium, and analyzed for ions and water (*12*). A minimal loss of potassium and limited gain of water were directly correlated with the healthy appearance of the tissue throughout the myocardium as observed during dissection. A total of four hearts analyzed after 7 and 8 days of perfusion and before the appearance of deterioration contained 318 ± 19 μmole of potassium and 3.84± 0.16 g of H_2O, compared to values for control hearts (not perfused) of 369 ± 26 μmole of potassium (N = 11) and 3.24 ± 0.11 g of H_2O (all figures are given per gram dry weight). Lack of swelling, nearly normal potassium content, satisfactory gross morphological appearance, and normal contractility are considered to be indices of normal histological, and electrical properties (*13*). It is significant to note that the conditions of perfusion do not lead to extensive swelling of the perfused organ, despite the absence of colloidal oncotic agents. This apparently extends the deviation from Starling's law of edema formation to hearts. Similar conclusions were previously reported for livers and kidneys (*14*).

Despite their apparent morphological integrity, there is no certainty that hearts preserved as described here would be capable of supporting life immediately after transplantation. However, reports that hearts preserved for 24 hours (*15*) and 3 days (*7*) in protein-free media were able to assume circulatory support are encouraging. Long-term perfusion of functioning organs at moderate hypothermia would permit continuous evaluation of organ integrity. Whereas the larger volumes of solution required for single-pass perfusion may be prohibitive for the preservation of larger organs over prolonged periods, the method may permit evaluation of organs preserved at deep hypothermia before transplantation. With future refinements it may also be possible to repair organs in vitro. The single-pass preservation method described here, besides extending the survival of functioning hearts in vitro, provides an anatomically defined and functionally stable test system in which numerous chemicals and conditions can be evaluated for their roles in myocardial integrity. The perfusion fluid will also be useful for the evaluation of drug and toxicant effects on the myocardium.

Table 2. Final perfusate composition.

Mineral salts and carbohydrates (mmole/liter)	
Calcium chloride	2.5
Dextrose	5.0
Magnesium sulfate	1.0
Potassium chloride	5.4
Sodium bicarbonate	25.0
Sodium chloride	121.0
Sodium phosphate, dibasic	0.9
Sodium phosphate, monobasic	1.5
Acetic acid, sodium salt	0.15
cis-Aconitic acid	0.04
cis-Oxalacetic acid	0.04
Citric acid	0.1
Fumaric acid	0.04
DL-Isocitric acid	0.04
α-Ketoglutaric acid	0.16
L-Lactic acid	0.5
D(+)-Malic acid	0.04
Pyruvic acid, sodium salt	0.14
Succinic acid	0.04
Polypeptide components (mg/liter)	
Bovine serum albumin	100.0
Insulin	0.056 (1.25 I.U.)
Glucagon	0.001
Amino acids, trace elements, vitamins, metabolic regulators, and lipids† (μmole/liter)*	
Copper sulfate	0.11
Ferrous sulfate	3.00
Manganic sulfate	4.00
Zinc sulfate	3.00
Adenosine	0.4
Adenosine triphosphate	10.0
p-Aminobenzoic acid	2.2
Ascorbic acid	100.0
Biotin	0.7
Choline citrate	11.0
Cytidine	0.2
Amino acids, trace elements, vitamins, metabolic regulators and lipids† (μmole/liter) (continued)*	
Folic acid	0.1
Guanosine	0.1
Hydrocortisone	0.16
Hypoxanthine	0.3
Inosine	0.1
meso-Inositol	40.0
Niacin	12.0
Pantothenic acid	1.4
Putrescine	1.0
Pyridoxal	2.4
Pyridoxine	0.8
Riboflavin	0.6
Thiamine	0.9
Thymidine	0.12
Uridine	0.2
Vitamin B_{12}	0.03
Calciferol	0.005
Cholesterol	0.22
Linoleic acid	0.146
Menadione	0.008
Palmitic acid	0.086
cis-Retinol	0.07
Thioctic acid	0.5
α-Tocopherol	0.05
α-Aminobutyric acid	20.0
Arginine	115.0
Asparagine	42.0
Citrulline	44.0
Cysteine	45.0
Glutamine	570.0
Glutathione	72.0
Glycine	360.0
Methionine	32.0
Ornithine	23.0
Proline	226.0
Serine	270.0
Tyrosine	120.0
Other (unit/liter)	
Penicillin G	100,000

*Other amino acids not listed here but present in rat plasma as analyzed by Scharff and Wool (*16*) were added at concentrations analyzed. †Fatty acids which may be associated with serum albumin are not included.

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Spinal Fluid Differences in Experimental Allergic Encephalomyelitis and Multiple Sclerosis

Abstract. *The spinal fluid of sheep with experimental allergic encephalomyelitis contains myelin basic protein (6 to 18 nanograms per milliliter) bound to antibody as well as excess free antibody. This bound myelin basic protein appeared concurrently with the onset of the disease and remained elevated until death. In contrast, in active multiple sclerosis, the spinal fluid contains free myelin basic protein and there are no detectable levels of antibody. The results indicate that the antibodies enter the spinal fluid from the serum by passive diffusion. This mechanism may also explain the presence of viral antibodies in the spinal fluid of multiple sclerosis patients.*

Experimental allergic encephalomyelitis (EAE) has been used as an animal model for multiple sclerosis (MS) (*1*). EAE is an autoimmune demyelinating disease induced in susceptible animals by immunizing them with tissue from the central nervous system or the basic protein of myelin, the antigen responsible for the immune reactions of the disease (*2*). We have examined myelin basic protein in the cerebrospinal fluid (CSF) of MS patients and have shown that, during episodes of acute exacerbation, myelin basic protein is released into the spinal fluid (*3*). These observations stimulated our interest in the dynamics of the release of myelin basic protein into spinal fluid during a defined demyelinating episode. The limitations in obtaining frequent serial samples of spinal fluid in humans and the unpredictability of the course of MS led us to study an animal model of demyelination.

Experimental allergic encephalomyelitis has been well established in sheep. The clinical sign of ataxia and paresis appear suddenly and are easily detectable (*4*). Animals usually develop EAE within 2 weeks after injection of a mixture of complete Freund's adjuvant emulsified with brain homogenate. Spinal fluid was obtained through an indwelling cannula over the cisterna magna (*5*). After implantation of the cannula, EAE was induced (*6*) and spinal fluid was withdrawn at frequent intervals from all animals. The spinal fluid was examined for cell counts, total protein, gamma globulin, basic protein (*7*), and antibodies to basic protein (*8*).

Eight animals were studied. Although all the animals were of the same breed and were treated in the same way, only half developed clinical evidence of EAE 8 to 20 days after immunization; the other half remained asymptomatic. This is similar to the results found by Panitch *et al.* (*4*) for this breed of sheep, which are outbred, and, as demonstrated by experiments with rats (*9*), the necessary determinants for EAE susceptibility are probably genetically controlled.

Studies of antibody to myelin basic protein in the spinal fluid from four animals with postmortem histological evidence of EAE are shown in Fig. 1A. In this group, antibody appeared in the CSF within 2 days of the onset of the disease. In contrast to MS, the spinal fluid contained high levels of antibodies to myelin basic protein which rose in parallel with serum antibody. The serum (not shown) contained 100 to 200 times more myelin basic protein antibody than the CSF (as measured by the reciprocal of the dilution that gave 50 percent binding of 1 ng of ^{125}I-labeled basic protein). These levels of CSF antibody could not be accounted for by leakage of blood into the spinal fluid as determined by cell counts of the CSF.

Surprisingly, the spinal fluid of the four animals without signs of the disease also contained increased amounts of antibody to myelin basic protein (Fig. 1B). All these animals were without postmortem histological evidence of EAE. The time of appearance and the amounts of antibodies in the spinal fluid were virtually undistinguishable from those in the group that did show EAE. These animals also had serum myelin basic protein antibodies in titers similar to those in the sheep with EAE.

We were unable to detect any free

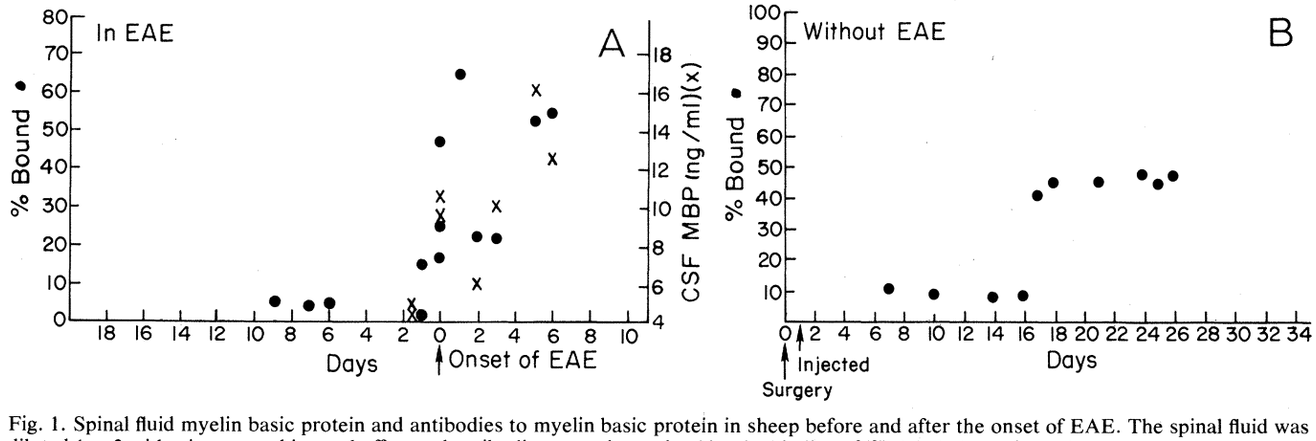

Fig. 1. Spinal fluid myelin basic protein and antibodies to myelin basic protein in sheep before and after the onset of EAE. The spinal fluid was diluted 1 to 3 with tris-acetate histone buffer, and antibodies were determined by the binding of ^{125}I-labeled myelin basic protein (*8*). Myelin basic protein was determined by radioimmunoassay (*7*). Data are expressed as percent of ^{125}I-labeled basic protein bound by the spinal fluid sample (•) and nanograms of myelin basic protein per milliliter of spinal fluid (x). Each point is the average of two values for each spinal fluid sample. (A) Levels of antibody from four animals and levels of myelin basic protein from three of these animals with EAE. In this group, cannulas were implanted between days 10 and 20, before the onset of EAE. They were immunized with human brain–complete Freund's adjuvant the following day. (B) Data from one animal representative of the group of four without EAE. This animal had a cannula implanted on day zero and was immunized on day 1.

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