SYMPOSIA

BONE MARROW TRANSPLANTATION AND RADIATION PROTECTION

Summaries of contributions by participants at Conference in Atlantic City, N. J. April 10, 1961

Co-chairmen: L. M. TOCANTINS and C. C. CONGDON

A. Experiments with Small Animals

G. F. Rabotti (Philadelphia) reported work on white blood cell and differential counts in mice exposed to radiation (500 to 900 r) and given treatment with isologous or homologous bone marrow or isologous spleen. A comparison was made on the rate and extent of recovery of the blood level of granulocytes, lymphocytes and monocytes for the different radiation doses and kinds of hematopoietic cell treatment. R. C. MacCardle (Bethesda) drew attention to the different kinds of plasma cells near the pancreas in mice, showing a wide range of variation in histologic and cytologic appearances. I. H. Wang (Buffalo) demonstrated that a mixture of AET, MEA, and serotonin as protective agents could give 84 per cent survival in mice irradiated at 1400 r. His control mice had an LD₅₀/30 days of 680 r; 6 x 10⁶ isologous bone marrow cells given alone to irradiated C3H mice resulted in an LD₀ value of 2000 r. A. L. Kretchmar (Oak Ridge) demonstrated the marked liver cell enlargement occurring in foreign bone marrow-treated mice and showed that this was associated with an increased nitrogen content of the organ. T. Makinoshan (Oak Ridge) reviewed work on the effect of irradiation on primary and secondary response of mice to red blood cell antigens, and showed that the two kinds of response differed only quantitatively. He also reported on the radiation sensitivity of antibody-forming cells in the in vivo tissue culture system, in which irradiated spleen cells and an antigen are injected into heavily irradiated mice. From the regression line of the log of the activity versus x-ray dose, he calculated the target size for the sensitive site in the cell. The estimate was 0.4 μ in diameter. From these and other data, estimates could be made of the total number of antibody-forming cells in a mouse and the radiation sensitivity of different immune functions, such as homograft rejection and humoral antibody production. The radiosensitivity of the homograft rejection mechanism was much less than that for humoral antibody production. I. L. Stcloff (Philadelphia) showed that though tetanus antitoxin production by fresh bone marrow in mice could be demonstrated, frozen preserved marrow did not give protection against tetanus toxin, indicating that the immune function of bone marrow was impaired by the preservation procedure. G. Cudkowicz (Oak Ridge) reported results on secondary disease in mice in which the homologous bone marrow donor mice were given 400 r in vivo, then killed immediately and their marrow infused into lethally irradiated recipients. There was a reduction in the amount of secondary disease. In addition, AET seemed not to protect immunologically competent cells. Parent liver killed sublethally irradiated F₁ recipients, but not if the parent liver donors were given 400 r before the livers were taken. AET did not protect this function in the irradiated liver. L. J. Cole (San Francisco) mentioned that similar experiments with irradiated marrow taken 12 days after exposure gave less secondary disease and J. J. Trentin (Houston), while doing this same type of experiment, failed to observe any effect from irradiation of the donor tissue. O. B. Zaalberg (Philadelphia) showed that sex-linked tissue incompatibility did not produce early death or secondary disease in irradiated bone marrow-treated mice even when female mice were sensitized against the Y antigens.

B. Viable Cells and Transplantation Antigens

E. A. McCulloch (Toronto) reviewed a method for producing clones of cells in the spleen of irradiated mice given small doses of bone marrow. Using this assay technic, he determined
the radiation sensitivity of marrow cells. Five per cent glycerol gave the best preservation of marrow cells, as measured by counting clones formed in the spleens of irradiated mice. J. E. Garvin (Chicago) described experiments on formed peripheral blood elements from heparinized blood passed through glass beads. Granulocytes and platelets stuck to the beads, and lymphocytes passed through. Temperature, magnesium and calcium ions influenced the separation of these cellular elements. F. Celada (Oak Ridge) reported that 100 cells were enough to preimmunize against homologous transplantation antigens. Dead cells were less effective than living cells. The loss of ability of homologous spleen cells to produce anti-sheep red blood cell agglutinins was used as the transplantation antigen assay system. J. Iossifides (Philadelphia) studied the effect of radiation on the antigenicity of skin transplants in mice. Skin isografts and homografts given 800 to 5000 r and transplanted 16–20 hours after irradiation took or were rejected in the expected manner. There appeared to be no effect on antigenicity in this test system.

C. Experiments with Large Animals

E. D. Thomas (Cooperstown) gave the results of experiments on irradiation and homologous bone marrow therapy in disease-free dogs. Radiation exposures were from 1200 to 1700 r at 5 r/min. with cobalt gamma rays. Some marrow-treated dogs recovered, as determined by peripheral white blood counts and platelets, but still died of infection. Some dogs rejected grafts. Others lived to 100 days after the experiment and died of secondary disease. Different dose schedules of Methotrexate were also used in some experiments to prevent secondary disease. Five dogs were maintained in good condition for an extended period of time in this manner. Cole (San Francisco) found that four doses of 6-mercaptopurine given at intervals the week before 900 r and 20 x 10⁶ homologous marrow cells promoted good marrow takes, but by that time the treated dogs were showing evidence of secondary disease. S. M. Michaelson (Bochroster) described the clinical and pathologic toxicity of AET in dogs. E. B. Hager (Boston) reported that a dog given 1500 r and autologous bone marrow after bilateral nephrectomy and kidney-grafting the day before, rejected the transplanted kidney on about day 12.

D. Clinical Results

W. B. Nycy (Seattle) described the case of a 7-year-old female child who developed aplastic anemia while on anticonvulsant therapy. Her identical twin sister was used as a bone marrow donor and 5–6 billion marrow cells were obtained from 44 puncture sites. A normal hematologic picture developed in time, but there was a period of hemolytic anemia and granulopenia during the rather remarkable recovery phase. Epilepsy returned, and anticonvulsant therapy had to be resumed. J. J. Trentin (Houston) mentioned a twin with aplastic anemia thought to be caused by chloramphenicol. The child was given bone marrow from an identical twin without benefit. D. G. Miller (New York) gave the results of transplantation studies on patients with malignant lymphoma. Skin and bone marrow grafts were tried. All showed delayed rejection times. Heterologous skin grafted on these patients had a long survival.

SPECIAL ASPECTS OF TISSUE TRANSPLANTATION, ESPECIALLY BONE MARROW PRESERVATION AND RADIATION PROTECTION

Summaries of presentations at Conference held in Buffalo, N. Y., August 18, 19, 1961.

Co-chairmen: E. Witebsky and A. P. Rinfret

Physical Mechanisms in Cell Death at Subzero Temperatures

P. Mazur, Oak Ridge National Laboratory, Oak Ridge, Tenn.

Cells exposed to subzero temperatures are subjected to two types of physical-chemical phenomena. On the one hand, molecular kinetic energy decreases and produces changes
in chemical reaction rates, diffusion rates, solubility, and viscosity. Secondly, water solidifies. Although changes in the first category (induced by the drop in temperature *per se*) could conceivably cause cell death, they do not do so with cells of the yeast *Saccharomyces cerevisiae*. Temperatures harmful to cells in frozen suspensions are entirely innocuous to cells in supercooled suspensions. Death is associated with ice crystal formation. Freezing subjects the cells to the direct effects of crystal formation but it also exposes them to high concentrations of solutes during the freezing process. Either could be detrimental to viability. Conceivably, the longer cells are exposed to harmful temperatures, the greater the injury. But in three species of microorganisms studied, longer exposures either reduce injury or have no effect. Slow cooling, for example, produces longer exposure to concentrating solutes but is considerably less harmful than rapid cooling. These findings apply both to cells suspended in deionized water and to cells in aqueous solutions of either electrolytes or nonelectrolytes. In fact, the presence of electrolytes in the surrounding medium actually reduces the damage from low temperature exposure. It seems that death is associated with direct effects of ice crystal formation and not the indirect effects of high solute concentration. A third factor involves the location of the lethal ice crystals (intracellular or extracellular). In these microorganisms, the intracellular ice formation seems to be the lethal factor. The fact that the cells can survive total extracellular freezing above certain temperatures, that slow cooling has a protective effect, the decreased volume of slowly cooled cells, and the microscopic appearance of frozen cells support this conclusion. It is uncertain how far these conclusions can be extended to other cells; knowledge of these mechanisms may make possible prevention of cell death by suitable pretreatment, additives, freezing procedure, or post-thawing treatment.

**FREEZING OF MITOCHONDRIA ISOLATED FROM RAT LIVER**

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Mitochondria were isolated in 0.44 M sucrose and the pH was initially adjusted to 6.2 with citric acid. These preparations retained most of the original structure, shape and size observed in situ and had very low dehydrogenase and ATPase activities. Mitochondria suspensions were frozen and stored, then thawed and fractionated by differential centrifugation. The fractions were examined in the electron microscope and analyzed for optical density, water content and various enzyme activities. The extent and kind of structural damage caused by the freeze-thaw procedure was described and related to specific enzyme tests. All damaged preparations had mitochondria at all stages of deterioration from normal structure to emptied and broken membrane material. Glutamic dehydrogenase activity was shown to be a measure of rupture and leaching, while β-hydroxybutyric dehydrogenase and ATPase activities reflect a change probably in permeability, not readily detectable in the electron microscope. By measuring enzyme activities, the damage caused by various freeze-thaw procedures was observed. No appreciable change was observed in mitochondria suspensions without at least half-hour storage in the frozen state. At temperatures above −40 C., most of the damage occurred after overnight storage, but maximum damage required up to three days’ storage depending on temperature. Rate of cooling and rewarming were not the major factors involved. Essentially no damage occurred at temperatures of −40 C. or lower, even after two months’ storage, provided the suspensions were thawed rapidly. At temperatures higher than −20 C., similar damage was obtained when mitochondria were suspended in sucrose solutions with a freezing point equal to the temperature of storage (i.e., no ice could form) and, after storage, diluted to 0.44 M sucrose. Less damage occurred when the storage took place at temperatures higher than the freezing point of the solution. Considerably less damage occurred when ATP was added before diluting the sample. These results are consistent with the assumption that ice crystals are not directly involved in the damage of mitochondria by freezing, and that the metabolic state of mitochondria is involved in their ability to withstand the osmotic shock of freezing (concentration) and thawing (dilution).
APPLICATION OF RESPONSE SURFACE TECHNICS TO THE PRESERVATION OF MAMMALIAN CELLS

Donald A. Gardiner, Oak Ridge National Laboratory, Oak Ridge, Tenn.

This paper describes research performed by Cragle et al. (J. Dairy Science 38:508, 1955) on the preservation by freezing of bovine spermatozoa. Emphasis is placed on the statistical technics used rather than on the results of the investigation. A three-dimensional central composite design in 15 treatments was laid out in the space of sodium citrate, glycerol, and equilibration time. Sperm from two ejaculates on each of four bulls was treated as called for by the experimental design and per cent motility after thawing was observed. Preliminary analysis indicated that the fitting of the response, \( Y \), (per cent motile) to the equation

\[
Y = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i<j=1}^{3} b_{ij} X_i X_j
\]

would yield useful information concerning the shape and orientation of the response surface. Additional analysis indicated that the surface could be roughly approximated by ellipsoidal contours centered at 2.9 per cent sodium citrate, 7.6 per cent glycerol and 14.9 hours equilibration time, with an estimated maximum motility of 67 per cent.

TOLERANCE OF GOLDFISH TO GRADUAL INCREASE OF AMBIENT GLYCEROL CONCENTRATION

Charles E. Huggins, Massachusetts General Hospital, Boston

Progressive addition of graded amounts of glycerol to water surrounding goldfish permitted equilibration at concentrations up to 1.35 M/liter. This is distinctly higher than tolerated strengths of substances not expected to cross the gill membrane, such as sucrose and sodium chloride. This difference plus that in toxicity between acute and chronic glycerolization indicates that the high concentrations are tolerated by equilibration of glycerol across the gill membrane. With glycerol concentrations greater than 0.4 M/liter, the fish exhibited a reversible neurologic abnormality manifested by swirling and loss of equilibrium. This ability of fish to absorb glycerol may help in attacking the problem of whole organ and animal preservation by freezing.

NEW FREEZE-DRIYING APPARATUS

M. Persidsky and V. Richards, Presbyterian Medical Center, San Francisco

A new, efficient freeze-drying apparatus has been designed incorporating (1) operation at high vacuum, (2) proximity of condensing surface and vacuum gauge to specimen, (3) large-diameter, short-length exhaust path aided by gravity, (4) long-term, continuous refrigeration of the condenser, and (5) controlled heating of the specimen. A vertical glass cylinder of large diameter contains a hollow metal cylinder with fins, refrigerated by aspiration of liquid nitrogen. The refrigerated metal cylinder serves both as a condenser and as a refrigerator, for the specimen is placed above it on a metal plate which is electrically heated while temperature is controlled by a thermostimulator. The plate heater is movable and can contact the refrigerated cylinder below or be raised up to melt paraffin, permitting vacuum-embedding of the specimen. As an alternative, the specimen can be placed above the condenser on the metal screen between two infrared heaters. A glass liquid nitrogen container forms a "cold-finger" top to the glass cylinder. The bottom is concave to augment the condensing surface. The glass cylinder at its lower end is connected to an oil-diffusion pump through a specially designed metal valve with a large opening. This valve is used to bypass the oil diffusion pump when the forepump is used at the beginning of the operation, and to permit continuous operation of the oil diffusion pump while the specimen is changed. A metal stopper containing an "O" ring is lowered inside the metal cylinder from its central portion with a large cavity into its narrower lower part with conical walls. Lowering and raising is by a still cable and crank. The bypass for the preliminary operation of the forepump is located above the metal stopper and can be closed by a glass valve. The thermo-
couple gauge is located above the glass valve and the ionization gauge is connected directly to the specimen chamber near the specimen, which is placed on the heating plate. As a modification, for large-scale, freeze-drying of tissue, six short glass test tubes surrounded by heaters to control drying can be mounted with the specimen in the refrigerated cylinder. These tubes can be sealed under vacuum by moveable stoppers. Control of ice sublimation and drying, sealing of specimens under vacuum, and continuous refrigeration are simply and uniquely attained.

**EFFECTS OF GLYCEROL AND DIMETHYL SULFOXIDE ON THE CAPILLARY CIRCULATION OF THE GOLDEN HAMSTER AND ON THE ISOLATED HEART**

Audrey U. Smith, National Institute for Medical Research, Mill Hill, London

The capillary circulation in the cheek-pouch and mesentery of the golden hamster has been studied by microscopy, and the effects of 15 per cent glycerol and of 15 and 30 per cent dimethyl sulfoxide observed. When glycerol is introduced into the peritoneal cavity or applied locally to a small area of mesentery, the capillary circulation is brought irreversibly to a standstill both locally and at a distance. By comparison, dimethyl sulfoxide causes comparatively little change. Determinations of corpuscular and plasma volumes and studies using serum albumin labeled with I\(^{131}\) indicate that the capillaries are relatively impermeable to glycerol which, therefore, exerts profound osmotic effects when administered parenterally. For example, intraperitoneal injection of glycerol causes withdrawal of water from the circulation and tissues with accumulation of fluid in the peritoneal cavity. Transfusion of glycerol, on the other hand, causes a striking increase in plasma volume. The capillary endothelium is more readily permeable to dimethyl sulfoxide which, when administered parenterally, causes slight but transitory changes in the distribution of water in the intact mammal. Isolated hamster hearts do not survive sudden increase or decrease in the concentration of glycerol in the fluid used to perfuse the coronary circulation, but will continue to beat if the concentration of glycerol is gradually raised over the course of two to three hours, and then very slowly reduced over three to four hours. By contrast, the concentration of dimethyl sulfoxide in the perfusion fluid can be rapidly raised to and lowered from 12 or 15 per cent without affecting the capacity of the cardiac muscle to contrast. Hamster hearts have survived freezing to temperatures between 0 and –20 C., both in the presence of 15 per cent glycerol and in the presence of 12 to 15 per cent dimethyl sulfoxide. We propose to continue these studies to establish the optimum conditions for long-term storage of the heart and other organs at low temperatures. Present indications are that dimethyl sulfoxide will be the protective additive of choice.

**IN VIVO AND IN VITRO VIABILITY TESTS ON FROZEN BONE MARROW**

I. Iossifides, Cardeza Foundation, Jefferson Medical College, Philadelphia

Preservation of mouse marrow by the slow-freezing method of Polges, Smith and Parkes is effective, as evidenced by the successful protection of lethally irradiated mice. When protective dose of 6 x 10\(^6\) to 12 x 10\(^8\) nucleated cells are used, no differences are seen in the recovery of mice protected isologously or homologously with either fresh or frozen bone marrow. In in vitro experiments, using short term cultures, suspensions of fresh marrow show an uptake of radioactive tracer proportional to the number of cells present and, for the first 20 hours, to the time of cultivation. Uptake of Fe\(^{59}\), C\(^{14}\)-glycine or C\(^{14}\)-sodium formate, S\(^{35}\) or H\(^3\)-thymidine is prompt, indicating functional and proliferative activity of the cells. Freezing, on the other hand, renders the cells unable to incorporate more than one per cent of the corresponding fresh aliquot. This was the experience with mouse, rabbit and human cell suspensions. On only one occasion, when rabbit bone marrow was frozen in homologous serum and 15 per cent glycerol, the uptake of the radioactive tracer varied from 8 per cent to 25 per cent, depending on the number of cultured cells. To investigate the possibility of a quantitative reduction of viable cells after freezing, consecutively lower numbers of fresh and frozen nucleated cells were used to protect lethally ir-
radiated animals. In first experiments, doses as low as $7 \times 10^6$ nucleated cells employed in isologous and homologous systems resulted in identical protection of lethally irradiated mice. There were no differences in recovery of the WBC, splenic colonization and mortality in animals protected with fresh or frozen cells.

**Low Temperature Preservation of Mouse Bone Marrow with Dimethyl Sulfoxide**

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Viability of mouse bone marrow cells stored at $-79\,\text{C.}$ with dimethyl sulfoxide was assessed by several in vitro and in vivo methods. Phase contrast, fluorescence microscopy with acridine orange and time-lapse cinemicrography, indicated that many cells frozen in the presence of 15 per cent dimethyl sulfoxide were intact. Biochemical studies on the protein- and lipid-synthesizing ability of these cells demonstrated that 10 per cent dimethyl sulfoxide gave the best protection. When lethally x-irradiated mice were injected with 5 $\times 10^6$ isologous cells, stored for one month at $-79\,\text{C.}$ in 15 per cent dimethyl sulfoxide, just over 80 per cent of the mice survived. Marrow preserved for one month in 15 per cent glycerol gave identical results with one important difference. Onset of reticulocytosis and recovery of the blood leukocyte levels occurred two days earlier in the group of mice treated with marrow preserved with 15 per cent dimethyl sulfoxide. Erythroid and myeloid stem cells seem to be better preserved with dimethyl sulfoxide than with glycerol.

**Preservation of Mouse Marrow**

*M. A. Bender and P. T. Tran*, Oak Ridge National Laboratory, Oak Ridge, Tenn.

Marrow from (C57BL x 101)F1 mice was preserved by freezing and later tested for ability to protect isologous mice irradiated with 900 r of x-rays. A total of $10^6$ cells were used per mouse, an amount producing about 75 per cent 30-day survivals with fresh marrow cells. Any drop in the number of viable cells in the thawed test suspensions was detected as a drop in the 30-day survival percentage. Slow freezing, by dropping the temperature of a commercial cooling bath 1 C./minute from 20 to $-25\,\text{C.}$ and then plunging the frozen sample into liquid nitrogen ($-196\,\text{C.}$), and fast thawing, by agitating the sample in a water bath at $37\,\text{C.}$, were the most successful methods. It was found that this method would not provide sufficient numbers of viable cells unless some protective compound was added to the suspension before freezing. Glycerol was found to be a very effective protective agent at 10 to 15 per cent concentration, yielding 30-day survivals of about 80 per cent. With glycerol, freezing and thawing can be done repeatedly if some loss of both cells and viability can be tolerated. It was found to be best to store the marrow at a high ($-30\,\text{C.}$) temperature between repeated freezings and thaws. Protective compounds were found among the polyalcohols, mono- and disaccharides, amino acids, and even inorganic salts. The best compounds, however, were either polyalcohols or monosaccharides. No attempts were made to remove these compounds after thawing. All were diluted by one-half and injected along with the marrow cells. Testing showed that none of the compounds promote recovery themselves. In cases where the solutions of protective compounds were not viscous enough to prevent clumping of cells during freezing and thawing, 3.5 per cent polyvinylpyrrolidone as used in addition to the protective agent. Long-term survival of marrow cells frozen in 15 per cent glycerol has been tested at $-30$, $-70$, and $-196\,\text{C.}$ When stored at $-30\,\text{C.}$, the marrow lost its ability to promote recovery by the 25th week. At $-70\,\text{C.}$, the marrow cells remaining unclumped were still effective after one year, but clumping had become extensive enough to make the suspensions of little practical value. The most recent test of the cells stored at $-196\,\text{C.}$, made at almost two to one-half years, gave just as good recovery as before freezing (83 per cent). In our experience, the most practical method for marrow storage is slow freezing in 15 per cent glycerol, storage in liquid nitrogen, fast thawing, dilution but not removal of the glycerol, and injection of the suspension, including the diluted glycerol.
SYMPOSIA

PRESERVATION OF BONE MARROW WITH POLYVINYLPRROLDONE

V. Richards and M. Persidsky, Presbyterian Medical Center, San Francisco

A suspension of rat marrow in a culture medium of one part of rat serum and three parts of Hank's solution was studied after exposure to different experimental preservatives. Freezing of marrow was accomplished by first cooling at the rate of 1 C./minute down to -25 C., then by carrying the temperature rapidly down to -79 C. in an alcohol bath, and maintaining this temperature for 10 minutes before thawing in a water bath at 38 C. Cell survival has been evaluated by phase microscopy after thawed cells are cultured on thin cover slips incubated at 37 C. in a mixture of Hank's solution and serum. Viable cells adhere to the cover slip, are usually elongated with projecting cellular processes, and appear bright in negative phase contrast illumination. Motility and contractility are occasionally observed. The number of live cells per microscopic field at 400 x magnification can be compared with control or untreated cells, using the latter as the 100 per cent figure in quantitating data on survival. A reliable quantitative method of assessing cell survival after various freezing technics with the use of various protective substances is thereby possible. Glycerol in 15 per cent concentration has been used as the "standard preservative." Sugars such as sorbitol, choline chloride, diverse molecular weights and concentrations of polyvinylpyrrolidone (PVP) and numerous molecular weights and concentrations of Carbowax have been evaluated. PVP in 10 per cent concentration of molecular weight 30,000 (Plasdone C) was the best preservative studied, being three to four times as effective as 15 per cent glycerol. Approximately 10 per cent of the cells survive under 15 per cent glycerol treatment, whereas 30 per cent appear viable under freezing with 10 per cent PVP. The Carbowax of molecular weight 400 to 600 also seems good, with about 30 per cent survival. The protective effect of PVP is probably related to its extracellular or surface-coating effects. It may have a binding quality with the lipoproteins of the cell membrane. Studies with 1131-labeled PVP (Raovin) have demonstrated that the majority of PVP can be removed from the cells after four washings with saline solution. The PVP is therefore extracellular and can be washed off from the surface readily. Our studies conclusively demonstrate that PVP in 10 per cent concentration is a better protective agent than 15 per cent glycerol in the preservation of rat marrow under slow freezing technics. A small amount of PVP may gain access to the interior of the cell by pinocytosis, a further protection of the cell against freezing. PVP also possesses the added advantage that it permits freeze-drying of the specimen, not possible with preservation under glycerol.

FREEZING EXPERIMENTS WITH RABBIT MARROW

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Studies on the preservation of bone marrow at liquid nitrogen temperatures were concerned with cooling and warming rates, protective additives, and the correlation of viability assays. Cooling rates were varied over an approximate 1000-fold range. Rates of 1 to 2 C./minute (0.03 C./second) were obtained using the Linde Controlled Rate Freezer. Rates of 0.3 C./second and 3 C./second were obtained in aluminum envelopes with liquid nitrogen vapor and liquid nitrogen, respectively. Rapid rates of 30 C./second were achieved by coating the envelopes before freezing in liquid nitrogen. All preparations were thawed rapidly at 37 C. Various additives were examined for their protective effect against freeze-thaw damage of bone marrow. Dimethyl sulfoxide was somewhat more effective than glycerol. Viability was measured by live-dead staining with both eosin and trypan blue. Rabbit serum alone also afforded protection to frozen and thawed bone marrow, as measured by this technic. Viability of marrow samples in different additives and additive combinations were not greatly altered with different cooling rates. The protein synthesizing capacity of bone marrow cells using C14-glycine was tested. Protective additives were checked for interference with incorporation of the amino acid into the protein. Serum enhanced the C14-glycine incorporation at low concentrations but not at high concentrations, presumably due
to dilution of the C¹⁴-glycine by free amino acids and peptides. Serum dialyzed to remove the smaller molecular weight fraction was not as effective as fresh serum in enhancing incorporation. Dimethyl sulfoxide in high concentration inhibited glycine incorporation and had to be removed to below three per cent before incorporation was similar to that of the controls. Marrow cells in 15 per cent dimethyl sulfoxide frozen in liquid nitrogen vapor and diluted after thawing to remove the DMSO were found to incorporate glycine into protein to approximately 80 per cent of the unfrozen controls, indicating that the metabolic integrity of the frozen and thawed bone marrow cells was preserved.

**Low Temperature Preservation of Canine and Human Marrow**

*J. W. Ferrebee and E. D. Thomas, Mary Imogene Bassett Hospital, Cooperstown, N. Y.*

Marrow was procured through a surgical “window” from the long bones of two dogs and frozen in 15 per cent glycerol by the technic of Polge, Smith and Parkes. One sample was kept at −80 C.; the other at −180 C. Fourteen months later the two dogs were given 1200 r of whole-body radiation from dual Co¹⁴ sources of (LD₁₀₀ = 600 r). The marrow was thawed rapidly, and the glycerol concentration reduced by the addition of 0.5 volume of 35 per cent dextrose followed by 2 volumes of 5 per cent dextrose. The marrow samples were then given intravenously to their respective donors. Both dogs recovered rapidly from the radiation and are normal six weeks later. A control dog given 1200 r but no marrow died after 11 days.

**Preservation of Human Marrow in Dimethyl Sulfoxide**

*Hugh M. Pyle and Hilda Boyer, Protein Foundation, Inc., Jamaica Plain, Mass.*

Dimethyl sulfoxide is a strongly hygroscopic non-viscous liquid with attributes that make it a valuable preservative for frozen tissues, particularly human marrow. The concentration of dimethyl sulfoxide is 15 per cent in Eagle’s tissue culture media adjusted to a pH of 7.8. Heparin is used as the anticoagulant. In vitro studies of human marrow preserved at −80 C. in this preservation media show an 85 per cent recovery of the bone marrow cells after prolonged periods of storage. The cells maintained their morphologic characteristics to a satisfactory degree. The primary morphologic change is that in the polymorphonuclear leukocytes; a lobulation to the segmented lobes and hyperchromia. The more immature myelocytes and red cell series maintain their morphologic characteristics to a satisfactory degree. Then marrow was incubated with tritiated thymidine following storage, autoradiographs showed 4.5 per cent tagging with tritium, the same percentage noted in nonpreserved marrow cells. Preservation toxicity studies reveal a loss of the administered dimethyl sulfoxide through the lungs and kidneys with no evident concentration in any specific organ, and clearance from the blood in one hour. In vivo use of preserved marrow in humans has shown no acute or chronic changes in liver function or kidney function.

**Experience in the Preservation of Human Marrow**

*D. E. Pegg, Westminster Hospital, London*

Aspirated human marrow was processed before storage in order to remove excess diluent and fat, and to reduce the material to an essentially single cell suspension using closed circuit technics designed to avoid bacterial contamination, and large-volume sieving apparatus convenient for clinical use. Fifteen per cent by volume of glycerol was added to the suspension which was then packaged in 25 ml. polythene ampoules. Cooling to −79 C. was carried out in two stages: at 1 C./minute to −15 C. and then at 5 to 10 C./minute to −30 C. and thereafter at not more than 10 C./minute. An apparatus to reproduce this cooling process accurately involved the principle of immersion in two stages of a partially insulated cooling vessel in an alcohol bath at −79 C. Side effects (pyrexia, hemoglobinuria and venous spasm) from the administration of the stored material have been few and transient. In vitro studies of the thawed material have emphasized the extreme fragility of the cells im-
medically after thawing, so that the precise technic used to examine them has a marked
effect on the results obtained. Thus, cell counts by phase contrast microscopy yield signifi-
cantly higher results than the conventional technics. Morphology is much better preserved
if the cells are filtered on to cellulose acetate membranes than if they are smeared on glass.
Cytochemical technics for the demonstration of enzymes and essential cell constituents
show only slightly reduced concentration after one or sometimes two years' storage, al-
though more severe reductions are seen after three years. The acridine orange test gives
convincing evidence of gradual deterioration throughout storage but the Trypan blue dye
permeability test gives more optimistic estimates of survival than other technics. Tissue
culture of stored marrow has been disappointing. Morphology is well-preserved and mito-
chondrial movement and slow locomotion are seen, but the activity falls far short of that in
cultures of fresh marrow.

BONE MARROW TRANSPLANTATION IN CANCER CHEMOTHERAPY

J. L. Ambrus, C. M. Ambrus, E. Feltz and L. Stutzman, Roswell Park Memorial Institute,
Buffalo, N. Y.

Autologous and homologous bone marrow transplantation was shown to protect Rhesus
monkeys against lethal doses of whole-body irradiation or intravenous nitrogen mustard.
No late disease was observed in the homologous bone marrow-protected group during an ob-
servation period of two years. Permanent take of donor-type marrow was not demonstrated
in this experiment. Such studies are currently in progress. Epinephrine and bacterial lipo-
polysaccharides were shown to produce leukocytosis in normal monkeys. The amount of
hemopoietic depression in bone marrow-injected and control groups did not differ statisti-
cally, although the groups given bone marrow recovered, whereas the control animals died
during the period of maximal hemopoietic depression. If, during the phase of maximal
hemopoietic depression, bacterial lipopolysaccharides or epinephrine was injected into the
animals, the controls had no significant leukocytosis, whereas the animals given bone mar-
row did. Irritating oils injected into subcutaneous granuloma pouches, caused the appear-
ance of inflammatory cells in the irradiated, bone marrow-treated animals, but no such
changes were seen in the irradiated controls. These experiments seem to indicate that the
transplanted marrow may not be able to produce enough white cells, initially, to increase
the circulating level, but may produce sufficient white cells to make it available to tissues,
and thus to cause survival of the animal. The presence of satisfactory bone marrow reserves
can be demonstrated with the lipopolysaccharide or epinephrine challenge test. This method
can also be used in demonstrating bone marrow takes, following autologous transplantation,
where immunologic markers are not available. This method proved to be useful in predicting
autologous bone marrow takes in clinical studies on the value of autologous bone marrow
transplantation in chemotherapeutic regimes.

STUDY OF BONE MARROW PRESERVATION AND AGED PLATELETS

Joseph J. McGovern, Massachusetts General Hospital, Boston

Aged platelets exhibit lactic and malic dehydrogenase activity during storage. Freezing of
bone marrow in liquid nitrogen appears to give far better morphologic preservation than
freezing at —70 C. The highest percentage of cells is preserved and the least damage to
the cells takes place when fine droplets of the material are sprayed onto a wide surface con-
tainer floating or liquid nitrogen. The use of heparin as the anticoagulant results in de-
struction of the majority of cells and distortion of the appearance of those cells remaining.
When Sequestrene is used as the anticoagulant, the cells remain intact, indentifiable, and
there is almost no loss of total number of cells. Clinical studies involving irradiation treat-
ment of acute leukemia show that patients can probably withstand the effect of 600 r if
sufficient amounts of fresh, normal autologous bone marrow is reinfused after irradiation.
The same may be true with stored viable, normal autologous marrow. Remissions are also
possible after 250 r of total-body irradiation without the use of bone marrow transplanta-
tion.
Present State of Platelet Preservation

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Viability of normal human platelets preserved at 4 C. in various media was studied by the use of autologous platelet concentrations labeled in vitro with Cr51. As a criterion of viability were used: (a) the survival time of the infused platelets and (b) the value of "maximum recovery", i.e., the maximum percentage of platelet radioactivity which could be recovered in the recipient's circulation after infusion. After three hours of storage at 4 C., a definite, but moderate reduction in viability was observed in almost all the experiments. When the time of storage was prolonged to 24 hours, platelet survival time was found to be reduced to three to four days (normal, eight to ten days), and the maximum recovery was only 30 to 50 per cent of the value observed with fresh platelets. No significant difference was seen whether the platelets were stored as whole blood, as platelet-rich plasma, or as platelet concentrates suspended in a plasma medium. When stored in saline alone, platelets lost their viability much more rapidly. Platelets suspended and stored for 24 hours in DAS-gelatin showed insignificant levels of viability. After 48 hours of storage in a plasma medium, platelet viability was found to be constantly reduced to minimal values. Viability of human platelets frozen in glycerol-plasma and stored for 24 hours at -75 C. was also studied. While the survival time of these platelets was six to eight days, the maximum recovery was only 10 to 30 per cent of the value observed with fresh, non-frozen platelets. Using a rabbit model in which platelets were labeled in vitro with Cr51 or in vivo with P32, the favorable influence of inorganic phosphate and of glucose on platelets preserved at 4 C. was demonstrated. Furthermore, the addition of inosine and adenine to a plasma medium produced an even greater improvement in viability of the preserved platelets. The best medium so far obtained for the preservation of human blood platelets at 4 C. is a medium containing plasma, glucose, phosphate, inosine and adenine. However, when preservation was prolonged to 48 hours or more, even in this medium platelet viability fell to very low levels.

Protection of the Intestinal Tract Against Radiation

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With the aid of S35-labeled AET, dose schedules were worked out which would allow perfusion of the intestinal tract through catheters placed in the duodenum under fluoroscopic guidance, during a period of whole-body radiation. This perfusion schedule produced significant AET levels in the intestines but only insignificant levels in other tissues or transplanted tumors of the host. Histologic studies indicated that perfusion of the intestinal tract with AET during irradiation prevents damage to the intestinal epithelium but does not prevent bone marrow depression and hemopoietic death. Perfusion of the intestinal tract with AET during 900 r whole-body irradiation, followed by isologous bone marrow transplantation, resulted in 90-day survival of 19 out of 20 Fisher rats. All irradiated controls and all animals treated with AET only, or bone marrow only, died within this period of time.

Blood Preservation and Blood Substitutes

A Symposium of the Edward Mallinckrodt, Jr., Foundation on Blood Preservation and Blood Substitutes was held at Endicott House, Dedham, Massachusetts, December 11 and 12, with 28 participants. Dr. Shields Warren served as General Chairman. The subjects and discussion leaders were:
The Physical Properties of Blood as a Hydrodynamic Fluid—Dr. John L. Oncley, Department of Biological Chemistry, Harvard Medical School
Blood Preservation by Physical Means—Dr. James L. Tullis, Protein Foundation
SYMPOSIA

BLOOD PRESERVATION BY CHEMICAL MEANS—Dr. Eugene P. Cronkite, Brookhaven National Laboratory

ADVANTAGES AND HANDICAPS OF PRESENTLY AVAILABLE BLOOD SUBSTITUTES—Dr. Scott N. Swisher, University of Rochester School of Medicine

DESIRABLE CHARACTERISTICS OF BLOOD SUBSTITUTES—Col. William H. Crosby, Chief, Department of Hematology, Walter Reed Army Institute of Research

Those participating were: Dr. Benjamin Alexander, Boston; Dr. William S. Beck, Boston; Dr. Kenneth M. Brinkhous, Chapel Hill, N. C.; Dr. James Butcher, Philadelphia; Dr. R. Keith Cannan, Washington, D. C.; Dr. Bradley E. Copeland, Boston; Dr. William Damehek, Boston; Dr. Louis K. Diamond, Boston; Dr. John T. Edsall, Cambridge; Dr. Joseph W. Ferreebee, Cooperstown, N. Y.; Dr. Clement A. Finch, Seattle; Dr. John G. Gibson, Boston; Dr. Sam T. Gibson, Washington; Dr. Leonard Hamilton, New York City; Capt. Lewis L. Haynes, Chelsea, Mass.; Dr. Harold B. Kenton, Boston; Dr. William C. Moloney, Boston; Dr. Edwin E. Osgood, Portland, Ore.; Dr. Howard G. Parker, Berkeley, Mass.; Dr. Robert B. Pennell, Jamaica Plain, Mass.; Dr. John B. Shapleigh, St. Louis; Dr. Max M. Strumia, Bryn Mawr, Pa.

Recent advances in the knowledge of hemic osmotic pressure and viscosity were related to problems of the blood vascular system.

The key to the flow of blood lies in the size and type of capillary network through which it must pass. The flow through the capillaries has a fairly flat front, the blood moving largely as a unit with slippage along the walls. A normal sedimentation rate of red blood cells is important for smooth flow. The sedimentation rate tends to be increased by most long molecules, as those of the plasma extenders.

The role of the plasma extenders (Dextran, albumin, PVP, etc.) is to provide a temporary filler for the vascular system in the event of hemorrhage or shock, to permit the body to function while blood itself is being replaced autogenously.

The plasma proteins in addition to maintaining proper osmotic balance and viscosity act significantly as binding agents; for example, serum albumin binds one molecule of fatty acid strongly, and 10 or more loosely. This helps to maintain proper suspension of fats in the blood. Blood fats supply important nutrient material to vital organs, particularly the heart, which derives over 70 per cent of its energy from fatty acids.

Of interest from the pharmaceutical standpoint is the fact that many drugs are bound by albumin which helps to prevent their too rapid excretion. Hormones such as insulin, thyroglobulin and others are also bound by blood proteins.

There is a moot question as to whether in order to obtain molecules of optimal size (about 35 Å) for use as blood substitutes, it is better to form the desired product by the splitting of pre-existing long molecules or by synthesis of smaller molecules to a polymer.

Albumin also plays a role in the binding of ions. One albumin ion normally binds eight chloride ions. On the other hand, it is a poor binder for calcium, only a 1:1 ratio.

Because of recent interest in the use of cold for anesthesia and in other fields, there was a consideration of the effects of lowered temperature on the blood. The cryoglobulins do not contribute much to the total properties of blood, so a drop in temperature has relatively little effect on whole blood.

The normal viscosity of blood has an index of three. Under certain pathologic states this can go up as high as ten. Fibrinogen is responsible for roughly 20 per cent of the total viscosity of the blood. Deviation in viscosity can be readily handled by the homeostatic mechanisms of the blood. In cases of plasma cell tumor, the macroglobulins are materially increased and this leads to increased viscosity of the blood.

Polyamino acids have potential value as blood expanders.

Reconstituted preserved plasma must be used rapidly, especially because the lipoprotein linkage has been broken down. In contrast to plasma, albumin can be frozen and thawed repeatedly without change. The major requirements in plasma expanders are freedom from antigenicity and homogeneity of molecular size. Since a blood substitute must fulfill a number of functions, there is no single measurement that can be used to establish desirable criteria.
Dextran is slightly antigenic. About four per cent of normal persons will react because of cross immunity. Dextran may slightly prolong bleeding time, but this is not serious or lasting. It rarely occurs if less than 1500 cc. of six per cent suspension are used. Dextran is less desirable to use because of the difficulty of obtaining proper and uniform molecular size. It was pointed out that there are now 6,000,000 units of Dextran in the national stockpile. Dextran maintains up to 50 per cent of its original restorative power for a period of 12 hours. It is slowly metabolized and the bulk is excreted in two to four weeks.

PVP is effective and cheap. However, it does persist in the liver. Owing to the risk of serum hepatitis from whole blood transfusions, it is quite possible that this might be more dangerous than utilization of Dextran or even PVP.

A number of plasma products are available. Four units of plasma are roughly equivalent to one unit of albumin. It was pointed out that despeciated bovine or equine plasma is used widely throughout the world.

There was extensive discussion of the preservation of blood by freezing, and the role of glycerol in the protection of red blood cells during the freezing and thawing processes. The method developed by the Protein Foundation and the Chelsea Naval Hospital and utilized at the Naval Hospital has proved of great value. The mean survival of transfused washed red cells preserved by glycerol and freezing in the recipient’s body is over 30 days. Glycerol is effective for successful freezing of washed red cells. Only one per cent of the blood cells are lost with each successive freezing and thawing.

It is probable that there is no one substance that can be used as a universal blood substitute and that it is necessary to choose from the available number for any specific case.

A statistical method for maintenance of satisfactory inventory of the diverse groups needed in blood banks was presented by Dr. Brinkhous. It was felt that this might well have wide value in civilian blood bank practice.

The plasma expanders are of very little use in civilian practice; their chief place is in emergencies. One of the basic questions is, “Can one improve the supply and lower the cost of human albumin?”

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