regard: the Uganda kob (*Adenota kob*) (*1*), the impala (*Aepyceros melampus*) (*2*), and the lechwe (*Kobus leche*) (*3*).

Gross examination of the tracts revealed that both ovaries ovulate: ovulation had occurred recently in the left ovary in 24 instances and in the right ovary in 15 instances—a nonsignificant difference. No sign of egg wastage or embryo resorption was observed.

Among the ten uteri in which there was no evidence of pregnancy, nine had right uterine horns clearly larger than the matching left horns.

GRAHAM CHILD

National Museum,
Bulawayo, Rhodesia

A. S. MOSSMAN

University College of Rhodesia and Nyasaland, Salisbury, Rhodesia

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9 June 1965

Preservation of Mammalian Cells in a Chemically Defined Medium and Dimethylsulfoxide

Abstract. *Three established mammalian cell lines (cat kidney, L, and HeLa cells), grown in suspension in a protein-free, chemically defined medium, were stored under liquid nitrogen in the defined medium fortified with 4 and 8 percent dimethylsulfoxide. After storage for 1 month the recovery viability was 86 percent, and cells showed normal growth upon reinoculation into the defined growth medium.*

In the past several years numerous freezing procedures have been described for the preservation of animal cells cultured in the presence of serum or protein hydrolyzates.

Evans *et al.* (*1*) have shown that human skin epithelium (NCTC 3075), monkey kidney epithelium (NCTC 3526, LLC-MK2), and Earle's strain L C3H mouse fibrosarcoma cells (NCTC 2071) could be successfully frozen and recovered from storage at the temperatures of liquid nitrogen in a serum-free medium with 6 or 12 percent glycerol. Their procedure for cell recovery required the important technique of progressive dilution to remove glycerol from the cells.

This report will describe a method for the preservation of mammalian cells in a medium completely free of serum or protein hydrolyzate and containing dimethylsulfoxide.

Three cell lines—cat kidney, L, and HeLa—were grown in Nagle's (*2*) chemically defined medium (without insulin). The medium contained the following in milligrams per liter. (i) Amino acids: L-arginine HCl, 100; L-cysteine HCl, 75; L-histidine HCl, 60; L-isoleucine, 150; L-leucine, 300; L-lysine HCl, 300; L-methionine, 60; L-phenylalanine, 120; L-threonine, 135; L-tryptophan, 60; L-tyrosine, 120; L-valine, 150; and L-glutamine, 450. (ii) Salts: NaCl, 7400; KCl, 400; $NaH_2PO_4 \cdot H_2O$, 100; $NaHCO_3$, 500; $CaCl_2 \cdot 2H_2O$, 265; and $MgCl_2 \cdot 6H_2O$, 275. (iii) Carbon sources: glucose, 1000; and sodium pyruvate, 110. (iv) Vitamins; D-biotin, 1.0; choline Cl, 1.0; folic acid, 1.0; niacinamide, 1.0; Ca pantothenate, 2.0; pyridoxal HCl, 1.0; thiamine HCl, 1.0; *i*-inositol, 1.0; riboflavin, 0.1; and B_{12}, 0.002. (v) Antibiotics and other constituents: methyl cellulose (15 centipoise), 1000; phenol red, 12.5; streptomycin, 100; and penicillin, 100,000 unit/liter. The cultures were incubated at 34° to 36°C in rubber-stoppered 2-liter bottles, each containing 500 ml of medium, on a shaker (New Brunswick Gyrotory) operating at 124 to 130 rev/min. Cell viability was determined by the trypan-blue dye-exclusion method. When the cell count had reached approximately 1.5×10^6 cells per milliliter, each culture was centrifuged at 1000 rev/min for 20 minutes, and the cells were resuspended in 72 ml of fresh growth medium to give a concentration of approximately 10^7 cells per milliliter. Portions (12 ml) of the pooled cells were then placed in six sterile, 60-ml prescription bottles and centrifuged at 1000 rev/min for 10 minutes. The supernatants were decanted and replaced with 12 ml of fresh medium containing dimethylsulfoxide (DMSO) in concentrations of 1 to 6 percent for cat kidney and L cells, and 4 to 10 percent for HeLa cells, in increments of 1 percent. The optimum concentration range of DMSO for satisfactory cell recovery had already been determined.

The cell suspension from each bottle was dispensed as 1-ml portions into 1.2-ml ampules with a 10-ml hypodermic syringe fitted with a 3.8-cm, 20-gauge needle. The ampules were immediately pull-sealed by an automatic ampule sealer (*3*) and then placed at 4°C for 20 to 30 minutes.

At each concentration of the sulfoxide three ampules were sampled for determination of zero-time viability. The remaining ampules were placed in the wire rack of the liquid nitrogen freezer (*4*) and frozen at a rate of 1 degree per minute to about −40°C. The frozen samples were placed immediately in aluminum racks for ampules, covered with cardboard tubes (*5*), and stored in a Linde liquid nitrogen refrigerator (LNR-35) at −196°C.

The viability of frozen cells was determined by removing three ampules for each sampling period and placing them immediately into an agitating water bath at 40°C. Suspensions were completely thawed in 30 to 55 seconds. The ampules were opened aseptically by scoring the neck with a file and snapping off the top. The thawed cell suspensions were aseptically transferred from the ampule to a 12-ml conical centrifuge tube containing 3 ml of

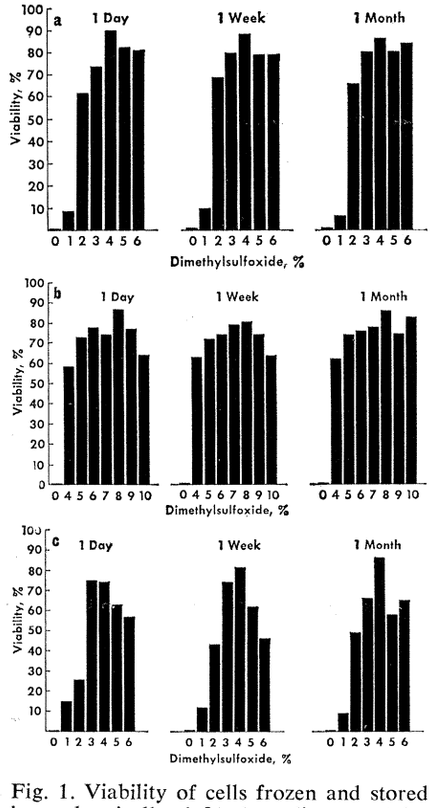

Fig. 1. Viability of cells frozen and stored in a chemically defined medium containing dimethylsulfoxide; (*a*) L cells, (*b*) HeLa cells, and (*c*) cat kidney cells.

fresh growth medium. The suspended cells were then centrifuged for about 10 minutes at approximately 1000 rev/min.

This process was repeated twice; after the second washing the cells were suspended in 25 ml of growth medium in a 100-ml serum bottle and the viability was determined. Cell suspensions were then placed on the shaker, the cells were counted daily, and the medium was changed at various intervals until a maximum cell population was reached.

L cells, continuously cultured in a serum-free system and frozen and stored as described, gave the highest recovery of viable cells (90, 88, and 86 percent) in medium containing 4 percent DMSO (Fig. 1*a*). Furthermore, at 3-, 5-, and 6-percent concentrations of DMSO, 80, 81, and 84 percent, respectively, of the cells were viable after storage for 1 month.

Cells frozen in 1 percent DMSO gave evidence of survival but failed to grow after incubation in fresh medium. The trypan-blue dye-exclusion method indicated that all cells were dead after incubation for 3 days. Cells frozen without DMSO appeared to be dead immediately after thawing.

Eight percent DMSO provided the most consistent viability values for HeLa cells (87, 81, and 86 percent) after storage (Fig. 1*b*). With cat kidney cells, 4 pecent DMSO showed the best viability (74, 81, and 86 percent) throughout the storage period (Fig. 1*c*).

The peak growth yields of L, HeLa, and cat kidney cells after storage in 4, 8, and 4 percent DMSO, respectively, for 1 month were 51.0, 47.0, and 41.0 $\times$ 10^5 cells per milliliter, respectively (Fig. 2). These growth curves compare favorably with those obtained with unfrozen controls.

Thus L and cat kidney cells frozen in 4 percent DMSO and HeLa cells frozen in 8 percent DMSO gave higher recovery than was observed with the other concentrations of DMSO, but they also grew in the presence of DMSO near these optimum concentrations. The epithelial-like HeLa cells required higher concentrations of DMSO for optimum recovery than did the fibroblast-like L and cat kidney cells.

We have shown that it is possible to grow and preserve mammalian cells suspended in a simple chemically

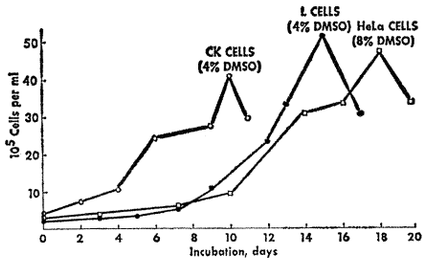

Fig. 2. Growth of L, HeLa, and cat kidney (*CK*) cells after storage in liquid nitrogen for 1 month at optimum concentration of dimethylsulfoxide (DMSO).

defined medium with DMSO as an additive in the complete absence of serum or serum products. The typical growth curves obtained from stored samples indicate that the conditions employed have not altered this important characteristic. These techniques might be applicable to the preservation of other materials (tissues, whole organs, and so forth) where protein-containing additives such as serum would be undesirable.

BRUCE L. BROWN
STANLEY C. NAGLE, JR.
U.S. Army Biological Laboratories, Fort Detrick, Frederick, Maryland 21701

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5. Frozen Semen Products, Division of Cryo-Therm, Inc., R.D. #1, Breinigsville, Pa.
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23 June 1965

Sulfur Dioxide in City Atmospheres

In "Air pollution affects pattern of photosynthesis in *Parmelia sulcata*, a corticolous lichen" [*Science* **148**, 1600 (1965)], L. Pearson and E. Skye report data (from a Warburg apparatus) which indicate that lichen disks in atmospheres contaminated by sulfur dioxide show "morphologic and photosynthetic abnormalities similar to those of lichens from an industrial center in Sweden." They suggest that "some kinds of lichens may be absent from city environments because of atmospheric pollutants which destroy chlorophyll." Although the authors are careful not to state unequivocally that the responsible atmospheric pollutant is sulfur dioxide, this is in fact the only pollutant studied in the report, and the inference is present that sulfur dioxide is in fact *the* pollutant. The concentrations of sulfur dioxide used in the experiments were 0.01, 0.05, 0.1, 0.5, 1, 5, and 10 percent by volume (uncorrected for solubility in the water present). Sulfur is present in polluted atmospheres primarily as sulfur dioxide, sulfur trioxide, and sulfate. In a study of ten cities during 1953–54 (Ann Arbor, Michigan; Akron, Ohio; Charleston, West Virginia; Cincinnati, Ohio; Detroit, Michigan; Elizabeth, New Jersey; St. Louis, Missouri; Washington, D.C.; and Whiting, Indiana), sulfur dioxide ranged from less than 0.01 to 0.38 parts per million by volume, with an average of 0.06; corresponding total sulfate values were less than 0.01 to 1.22, with an average of 0.10 [J. Cholak, L. J. Schafer, W. J. Younker, D. Yeager, *A.M.A. Arch. Ind. Health* **11**, 280 (1955)]. These concentrations appear to be in the same range as similar data from other cities throughout the world. Effluent flue gases from coal-burning plants typically have sulfur dioxide concentrations of 0.1 to 0.3 percent by volume. Pearson and Skye's experimental conditions represent sulfur dioxide concentrations of 100 to 100,000 parts per million, values which range from about 260 to 260,000 times larger than the maximum concentrations actually found in urban atmospheres. The data shown in Pearson and Skye's Fig. 1 are for the highest sulfur dioxide concentration (100,000 parts per million), which leads one to suspect that their highest concentrations are required for significant effects. Atmospheric pollution is a complex problem of growing, worldwide importance. Because of the economic and political problems connected with it, scientists should be especially careful to present their results so as to avoid unwarranted conclusions. This could have been done in Pearson and Skye's report by including a comparison of the sulfur dioxide concentrations used in their experiments with typical concentrations found in polluted atmospheres.

ROBERT H. LINNELL
R.D.#3, Gaithersburg, Maryland

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