Table 1. Multiplication of cells released from trunks, either normal or treated with puromycin; eight trunks were used in each series.

Incubation period (hr)	Cells ($\times 10^6$) from trunks:	
	Normal	Treated with puromycin
0	2.06	1.89
48	4.20	4.76
72	12.22	12.20

ed, as with the CSA labeled with sulfate.

Considerable variation in the degree of inhibition of incorporation of sulfate was observed for the lowest concentration of puromycin tested; the 26-percent inhibition (Fig. 1) represents the average of values from six experiments, which ranged from 13- to 46-percent inhibition. This fluctuation was not encountered with incorporation of either glucose or valine at this same concentration of puromycin, nor with incorporation of sulfate at the higher concentrations of puromycin tested; this discrepancy remains unexplained. The maximum degree of inhibition of synthesis of CSA requires about one-fifth the concentration of puromycin necessary for the complete inhibition of incorporation of valine.

The inhibition of synthesis of both protein and CSA is reversible (Fig. 2). When trunks are removed to puromycin-free medium, incorporation of valine into protein returns to a rate about 80 percent of normal, with gradual recovery of incorporation of sulfate and glucose into CSA.

The reversible nature of inhibition by puromycin has been observed in vivo (*8*), in cell-free systems (*9*), and in tissue slices (*10*). The release from the inhibition by puromycin, as shown by the return to the near-normal rate of incorporation of valine, does not in itself necessarily mean that these cells have survived this treatment. In order to demonstrate that cells in the trunks treated with puromycin could still be induced to synthesize DNA and all components necessary for cell multiplication, trunks were incubated in MEM in the presence and absence of puromycin; the same amount of tissue was obtained for each series so that the total number of viable cells could be estimated. After being incubated for 5 hours, they were transferred to puromycin-free MEM and incubated overnight. The next day they were treated with trypsin. The cells were then suspended, washed, and resuspended in 2 ml of medium, and 1 ml of the suspension was plated on clots and incubated for 48 and 72 hours, at which times the cells were recovered and counted. Almost the same number of cells (91 percent) were obtained (Table 1) from puromycin-treated trunks as from untreated trunks, and treated cells multiplied as well as normal cells.

Treatment of 10-day-old trunks with puromycin for 5 hours under the above conditions thus has no deleterious effect on the capacity of chondrocytes for cell division. However, treatment with puromycin beyond 5 hours under such conditions kills many cells: 70 percent are killed after a 6-hour exposure to puromycin.

Our results indicate that syntheses of CSA and protein are intimately associated. This idea is supported by the observation that actidione, another inhibitor of protein synthesis (*11*), inhibits synthesis of CSA (*12*). Since CSA is covalently linked to protein (*13*), it seems that its synthesis must be coupled to that of protein. This may also imply the existence of a feedback control of the synthesis of this polysaccharide whereby no protein-free CSA accumulates because it inhibits some step in its own synthesis. A recent report of a similar effect of puromycin on the synthesis of glycoprotein in rat liver is that inhibition of synthesis of protein led to the accumulation of uridine-diphospho-*N*-acetylglucosamine, which is an inhibitor of the first reaction in its own biosynthesis (*14*).

The long-term effect on CSA synthesis of a brief treatment of these cells with puromycin at a concentration that completely inhibits synthesis of protein is of some interest. The gradual return to normal synthesis of CSA, as determined by incorporation of sulfate and glucose, occurs only when the enriched medium is used; when trunks are instead transferred to MEM, a loss of 60 percent in the rate of incorporation of glucose or sulfate into CSA is still observed after 48 hours, whereas incorporation of valine returns to 70 percent of normal.

This failure to synthesize CSA may reflect a partial loss of some enzyme or enzymes in this pathway because of turnover during the inhibitory period, of accumulation of some inhibitor which affects synthesis of CSA more than protein synthesis, or of the loss of some cofactor necessary in this synthesis. It is significant that puromycin does not interfere with the release of radioleucine from labeled protein in liver slices (*12*), which fact is evidence for the turnover of protein (*15*); it has also been demonstrated that puromycin prevents neither the disappearance of tryptophan pyrrolase in rat liver when injected after full induction of this enzyme has been obtained (*16*), nor the turnover of rat liver amylase (*17*).

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Right Horn Implantation in the Common Duiker

Abstract. *In the common duiker,* Sylvicapra grimmia, *implantation occurs only in the right uterine horn, but both ovaries ovulate.*

Reproductive tracts of 49 female common duikers (*Sylvicapra grimmia*) were collected in Bechuanaland and Rhodesia. In 39 of these tracts pregnancy was obvious and the single embryo was implanted in the right uterine horn. Thus the common duiker resembles three other African bovids in this

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.

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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

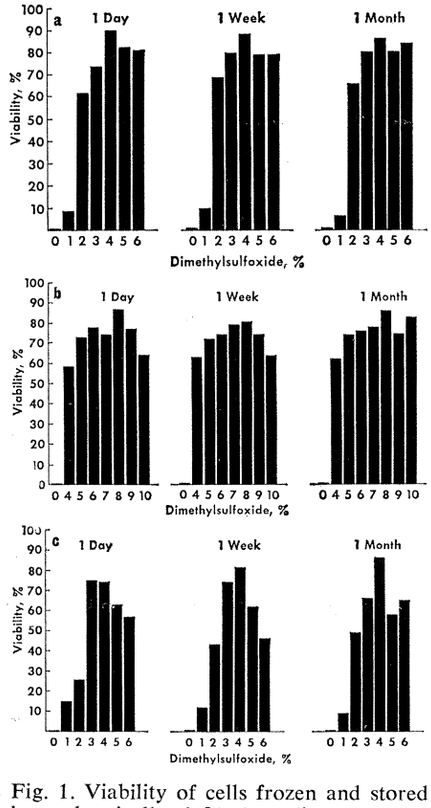

Fig. 1. Viability of cells frozen and stored in a chemically defined medium containing dimethylsulfoxide; (*a*) L cells, (*b*) HeLa cells, and (*c*) cat kidney cells.