mals(1,2), as bile of usual experimental animals can hold more cholesterol in solution than is normally present. On the other hand, human bile is more saturated with cholesterol (3). In our experiment we found that human gallstones are much less soluble in monkeys than in usual experimental animals (Table I), suggesting that monkey bile is more saturated with cholesterol.

There have been few reports indicating incidence of gallstones in monkeys(10). However, it is doubtful that gallstones in monkeys are identical to those of man, which contain a large amount of cholesterol. Lapin and Yakovlava(11) stated they found coagulated gall in the form of cholesterin pigmented clots or cholesterol sand. Fox(12) reported finding pigmented gallstones in the pig-tailed monkey (*Macacus nemestrinus*) but did not analyze the stone. Thudichum(13) stated that gallstones of monkeys are similar to those of man, but he also included pigs, which are now known to have stones of lithocholic acid (14).

Summary. 1. Human gallstones placed in monkey gallbladders are less soluble than those placed in other experimental animals. 2. The major constituents in monkey bile are compared with those in human and dog bile.

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Routine Development of Permanent Strains of Fibroblasts from Bone Marrow of Adult Rabbits. (25732)

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Permanent cell lines of fibroblasts have been established *in vitro* from fetal and adult rabbit tissues, but development of these lines was accomplished only after many failures (1). During studies designed to develop methods for continuous growth and maturation of rabbit bone marrow cells *in vitro* permanent strains of rapidly multiplying fibroblasts could be routinely established from explants of adult rabbit bone marrow. This report describes the relatively simple method used for routine development of permanent strains of fibroblasts from rabbit bone marrow and some growth and morphologic characteristics by these cell lines.

Material and methods. Six-week to one-year-old rabbits were anesthetized with nembutal and a femur was resected. The femur was grasped between 2 large hemostats and cracked with a twisting motion and marrow was carefully removed with No. 11 Bard Parker blade attached to No. 7 holder and placed in a 50 mm Petri dish in 5 ml of culture medium composed of 1 part calf serum, 2 parts medium 199(2) and 0.1% yeast extract (Difco). 400 Units of penicillin/ml and 80 μg/ml of streptomycin were routinely

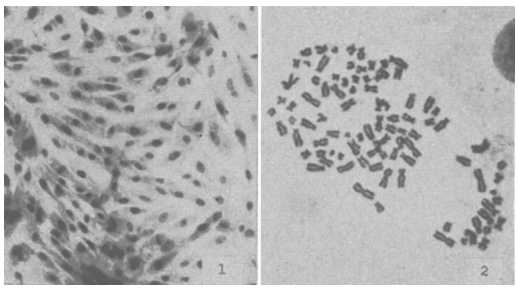

FIG. 1. Rabbit bone marrow strain RBM-1-G fibroblasts, 4 yr in continuous culture. Lillie fixation H. and E., ×50.

FIG. 2. Highly aneuploid RBM-1-G cell with 95 chromosomes, one yr after explantation. Hypotonic medium spread—Acetic orcein, ×800.

added to the medium. The marrow was pumped 4 or 5 times through a 20 gauge needle attached to 5 ml syringe; final suspension contained many small clumps of bone marrow particles as well as isolated bone marrow and peripheral blood cells. 0.3 ml of bone marrow suspension and 5 ml of growth medium were added to each of a series of 12 to 15 T-30 culture flasks, sealed with rubber stoppers and incubated at 36°C. The cells were subcultured by trypsinizing with a 0.05% solution of crystallized trypsin (Worthington Biochemical) in medium 199. When detailed cytologic studies were made, cultures were explanted on standard 3 x 1 inch microscope slides and grown in 8 ml of culture medium in rubber stoppered 6 oz square french bottles.

Results. Most small particles of bone marrow attached to the surface of T-30 flasks within 24 hours. Within 72 to 96 hours after cultures were made a dense outgrowth of spindle shaped cells developed from attached marrow particles. These spindle cells (Fig. 1) multiplied rapidly, so that at end of 10 to 14 days there was a monolayer of fibroblasts covering entire surface of flask. Ten to 15 minutes incubation in trypsin solution was needed to remove cells from the glass at the first and second subcultures, but thereafter they came loose within 5 minutes incubation at 36°C, as quickly as HeLa cells. Cells were subcultured at 5 to 7 day intervals with 2-3 x 10^5 cells added to each new T-30 flask during first 12 weeks of *in vitro* culture. As long as this number of cells was added at each subculture and pH of medium maintained between 6.9 and 7.6, by daily refeeding if necessary, there was no period when cell multiplication stopped. Although rate of multiplication declined during lag period which developed between 10th and 12th weeks, there was very little cellular degeneration. Towards end of lag phase, which lasted about 2 weeks, mitotic figures appeared in many areas of flasks, and this again resulted in formation of a dense monolayer of fibroblasts. After the lag phase, cell strains multiplied with average generation time of about 28 hours and were subcultured in T-30 flasks with 2-5 x 10^4 cells.

Rabbit bone marrow lines were maintained in continuous culture for over 4½ years and during this time fibroblast cells have maintained their spindle shaped morphology.

During the first 6-7 months of growth *in vitro,* there were many cells which contained the normal diploid (44) or near diploid number of chromosomes. After one year *in vitro,* no mitotic figures were observed which had the normal chromosome number and in addition new chromosome types had developed (Fig. 2).

Cells from cultures of fibroblasts derived from rabbit bone marrow have been preserved by both slow and rapid freezing and stored at −76°C in growth media containing 10% glycerol(3). These cells tolerate both slow and rapid freezing equally well with recovery of over 90% of cells originally frozen. Cells frozen rapidly showed an initial lag of 3 or 4 days before they began to multiply rapidly, while those frozen slowly began to multiply 24 hr after they were reexplanted. These rabbit cells tolerate rapid freezing better than those of human or murine origin grown under similar conditions.

Discussion. Permanent lines of cells established by Berman *et al.* from human bone marrow were reported to change in morphology from "fibroblast-like" to "epithelium-like" form during cultivation *in vitro*(4). This morphologic transformation did not occur during establishment of 13 lines of fibroblasts derived from rabbit bone marrow. Cell lines carried in continuous culture for almost 5 years have retained their spindle shaped appearance.

Similar strains of fibroblasts have been established from adult rabbit, spleen, testis and ovary. Epithelial strains have also been isolated from liver and ovary and other tissues containing epithelial cells but not from bone marrow. Epithelial strains were isolated from tissues explanted in plasma clot cultures.

Haff and Swim(1) found that 199 did not support growth of rabbit fibroblast lines. In this laboratory the combination of 199, calf serum and yeast extract has proved an ideal medium for rapid and continuous growth of rabbit fibroblast strains developed from a variety of adult rabbit tissues. In preliminary experiments it was observed that addition of yeast extract increased growth rate and also shortened the lag phase. However, once the lag period was overcome, cell lines derived from marrow and other rabbit tissues proliferate rapidly without yeast extract.

Routine development of fibroblast strains is making possible investigations into possible conversion of normal rabbit fibroblast cells into malignant ones *in vitro*. It is now possible to use the same rabbit for donor tissue and as a recipient after its cells have been grown for any given length of time *in vitro*. The relatively long life span of the rabbit (5 years) makes it another important laboratory animal in which to study mechanisms involved in conversion of normal into malignant cells *in vitro*.

Summary. A simple method is described for routine development of permanent strains of fibroblasts from adult rabbit bone marrow. Marrow particles were grown on glass in medium composed of 1 part calf serum, 2 parts 199 and 0.1% yeast extract. This method is also applicable to development of permanent cell lines from a variety of adult rabbit tissues.

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Non-Specificity of Pituitary-Induced Anuran Ovulation *in vitro*.* (25733)

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Ovulation in *Rana pipiens* induced by injection or implantation of frog pituitary glands is a common laboratory procedure. Implantations or extracts of pituitary glands of a few other vertebrates are also effective in *Rana pipiens, i.e.,* garpike(1), toad(2), newt(3), chicken(4), and sheep(5). Some other anurans, particularly *Xenopus laevis* and several urodeles, ovulate upon exposure to pituitary factors from a wider variety of animals or even pregnant mares' serum and human chorionic gonadotropin(6). In many instances, however, enormous dosages of heteroplastic pituitary tissue are required, suggesting that responses obtained may not be entirely physiological, or that injected material may have merely activated recipient's pituitary gland. Induction of ovulation *in vitro* by addition of extraspecific pituitary factors to Ringer's solution containing ovarian tissue from normal, healthy frogs, rules out participation by the host's pituitary gland, thus providing a very critical test. Routine induction of ovulation *in vitro* with frog pituitary extracts and/or preparations of FSH and LH from sheep pituitary glands has been reported(5,7). It now seems important to record ovulatory responses *in vitro* in a number of interspecific combinations of ovarian tissue and pituitary glands, not only to add data for consideration in the species-specificity controversy, but in view of objection of some(8) that ovulation *in vitro* may

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