liquid form was modified by dietary composition including high NaCl levels. Diethylstilbestrol injections also caused hyperproteinemia, hypercholesterolemia, hyperlipemia and lowered blood pressure. High NaCl diets did not affect blood pressure.

The technical assistance of J. W. Carlisle and C. J. Miller is acknowledged.

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Received March 4, 1964. P.S.E.B.M., 1964, v116.

Follicular Fluid Electrolytes in Ovulation.* (29241)

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Although many aspects of the composition of follicular fluid have attracted research interest, the electrolyte composition has been inadequately reported. In the present study Na and K concentrations in the fluid of rabbit follicles were determined at 3 stages of follicular development. These data permit evaluation of the hypothesis that imbalances in cation transport across the follicle wall occur in the ovulatory process(1).

Materials and methods. Ten sexually mature female rabbits of mixed strains were grouped as follows: Group I, 2 animals judged to be in diestrus on the basis of vaginal appearance and refusal to copulate; Group II, 4 animals classed as being in estrus on the basis of tumescent reddish-purple vaginal lining; Group III, 4 animals judged to be in estrus mated with normal males. All animals were lightly anesthetized (approximately 35 mg pentobarbital per kg i.v.), and opened with bilateral flank incisions for visualization of the ovaries. Mated animals were anesthetized 7 to 8½ hours after mating and sampled during the 9th hour.

The exteriorized ovaries were held in place by clamping the mesovaria parallel to the ovary, in a hemostat equipped at the tip with a spacer which prevented the jaws from closing on the mesovarian vessels. A sample of follicular fluid was taken from each of the large follicles; in the case of unmated estrus rabbits these were follicles which appeared to be sufficiently mature to ovulate if the animals had been mated (avg. diam. 1,600 ± 38 μ), in the case of mated animals the follicles sampled were those in which ovulation appeared imminent (avg. diam. 1,880 ± 62 μ). The largest follicles of the animals in diestrus were also sampled, although they appeared smaller, tougher, and flatter (avg. diam. 1,420 ± 56 μ) than those of the other groups. The sampling procedure was as follows: The follicle to be sampled was punctured by a tared micropipette formed from capillary tubing (1.5 mm OD) ground to a bevel (Fig. 1). With the aid of an aspirator, fluid was drawn into the pipette and it was reweighed. Sample weights ranged from .9 to 3.5 mg and could be reproducibly read to within ± .01 mg. Samples contaminated with blood were not used. After weighing, the pipette was rinsed into 8-9 ml of distilled H_2O in a 10 ml volumetric flask, .5 ml Li_2SO_4 solution was added and the volume brought to 10 ml with distilled H_2O. Plasma samples

* This work was supported by a grant from Nat. Inst. of Health.

TABLE I. Electrolyte Analyses of Follicular Fluid.

	Rabbit No.	No. of follicles	mEq K/kg fluid	No. of follicles	mEq Na/kg fluid
Group I. Unstimulated follicles (diestrus)	113	4	17.8 ± 3.4	4	170 ± 3.8
	117	6	10.3 ± 1.2	6	173 ± 5.1
Group means		10	11.3 ± 3.0	10	171 ± 3.2
Group II. Unstimulated follicles (estrus)	106	8	10.8 ± 2.9	8	152 ± 4.2
	108	8	7.8 ± .50	7	167 ± 4.8
	112	9	10.1 ± 1.8	10	153 ± 4.8
	115	9	5.9 ± .27	9	157 ± 4.5
Group means		34	8.6 ± 1.7	34	157 ± 2.4
Group III. Pre-ovulatory follicles	109	8	6.2 ± 3.1	8	150 ± 2.0
	111	9	10.0 ± 1.1	9	149 ± 2.1
	114	9	7.4 ± .97	9	149 ± 2.6
	118	6	6.3 ± .92	5	158 ± 6.5
Group means		32	7.6 ± .56	31	151 ± 1.5

Variability is expressed as SEM.

were prepared similarly from whole blood which had been drawn into heparinized tubes and centrifuged.

Electrolyte analysis was by internal standard flame photometer (NIL model equipped with photo-resistive cells). A final Li-concentration of 1.44 mEq/liter was selected as optimal for these small samples, since it is low enough for high sensitivity but high enough to confer the features of stability and reproducibility of the internal standard principle. The follicular fluid samples in each group analyzed were read against standard curves constructed from readings on solutions of known Na-K composition analyzed before, after, and interspersed among analyses of the unknowns. The analytical procedures were sufficiently sensitive to permit determination of the Na and K of a single follicle and thus calculation of the variation among comparably mature follicles of a single animal could be made.

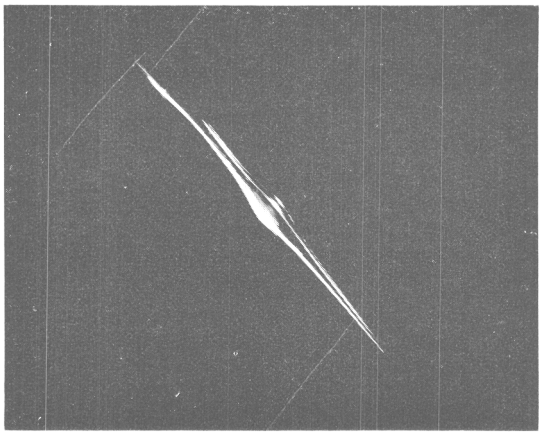

FIG. 1. Sampling pipette. Tips, 150-300 μ in diameter, are ground to a bevel on a diamond dental drill.

Results. Data for Na and K in follicular fluid are given in Table I. In all animals and in all groups of animals, variability is much greater among K than among Na determinations, and is sufficiently great that no differences in K concentration at the 3 levels of follicular development can be distinguished. The mean K concentration of all follicular fluid is 8.5 ± .5 mEq/kg and is probably higher ($p<.025$) than that of normal plasma (6.0 ± .56 mEq/kg, $n = 15$).

Na determinations were reproducible and consistent among the follicles of a single animal and among the animals of each group. Na concentration in follicles of rabbits thought to be in estrus was lower ($p<.0005$) than in diestrus animals. Na concentration in pre-ovulatory follicles was still lower ($p<.01$), 3.8% less than that in unstimulated, presumptively estrus, follicles. Although Na concentration in diestrous follicles was somewhat higher than the plasma concentration of 165 ± 2.04 mEq/kg ($n = 14$, $p<.025$), the concentration in the fluid of the estrous and pre-ovulatory follicles was lower than that of the plasma ($p<.01$ and $p<.0005$, respectively).

Discussion. The Na and K composition of follicular fluid is much more like that of

lymph or plasma than of intracellular fluid, as might be expected on the basis of the similarity of its protein composition to that of plasma and the apparent ready permeability of the barrier between capillary and follicle(2,3).

Measurements of intrafollicular hydrostatic pressure in rats and in rabbits indicate that in both unstimulated and pre-ovulatory follicles(4,5,6) pressures are within the range of normal capillary pressures. In view of the free exchange of H_2O between the follicular antrum and thecal capillaries, these measurements effectively rule out the existence of osmotic pressure gradients during follicular development. Consequently, variations in cation content between follicular fluid and plasma, and variations in the fluid at different stages of development, must be compensated by a) complimentary variations in other osmotically active constituents, or b) variations in the quantity of cation held in osmotically inactive, "bound" form. The composition of follicular fluid has not been sufficiently analyzed to rule out the possibility that some osmotic constituents vary inversely with the cation. Although protein analyses indicate that both albumin and globulin concentrations are somewhat lower in follicular fluid than in plasma(2,7), this deficit is quantitatively inadequate to account for the higher-than-plasma Na values in diestrous follicles and in the wrong direction to account for the lower Na values in estrous and pre-ovulatory follicles. Donnan effects might account for the inequality of cation distribution, but if so, this would require a rather large change in degree of ionization of the polyionic constituents of the follicular fluid as the follicle matures.

Cationic binding is perhaps the more attractive explanation of both the differences between plasma and follicular fluid concentration and of the change in content as the follicle swells and approaches ovulation. Mucopolysaccharide has been shown to influence cationic distribution in isolated systems(8), has been suspected of influencing electrolyte distribution *in vivo*(9), and has been shown to be a significant constituent of follicular fluid(10). Histological studies and measurements of viscosity of follicular fluid suggest that sulfated mucopolysaccharides are prominent constituents, and that as ovulation approaches depolymerization occurs(10,11). A polymer of chondroitin sulfate of n units holds approximately n cations in non-ionized form. With the cleavage of each unit, when associated with methylation of the carboxyl group or when desulfated, one "binding" site may be lost(8). Consequently, pre-ovulatory depolymerization of follicular mucopolysaccharide could be expected to result in a maximum reduction of cation concentration of 1 unit per unit cleaved from the polymer on an osmotic basis, and 1 unit lost from the "bound" fraction. Thus a pre-ovulatory depolymerization of mucopolysaccharides sufficient to increase osmotic pressure 40 mmHg, as suggested by Zachariae(10), would be sufficient to osmotically replace and to release from binding sites a total of 4.14 mOM Na/kg. The average measured difference in total Na between estrus and pre-ovulatory follicles was 6 mEq/kg.

The fact that Na concentration falls as ovulation approaches suggests that changes in electrolyte distribution, which might be caused by changes in steroid secretory patterns of the granulosa, artificially induced by steroid adjuvants of ovulation(12), or blocked by steroid inhibitors acting directly on the ovary(13), do not play an osmotic role in the mechanisms of ovulation. It seems that the changes in Na content associated with ovulation are at present best explained as a product, rather than a cause, of pre-ovulatory growth.

Conclusions. The Na and K concentration of follicular fluid is similar to that of plasma. The K concentrations of diestrus, estrus, and pre-ovulatory rabbit follicles are not measurably different. Average [K] values are slightly above those for normal plasma. Na concentration of diestrous follicles was somewhat higher than that of plasma, whereas the Na content of fluid from estrous and pre-ovulatory follicles was somewhat lower.

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Received February 13, 1964. P.S.E.B.M., 1964, v116.

A Simplified Method for the Cloning of Heteroploid and Diploid Mammalian Cells.* (29242)

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To study metabolic or morphologic differences between individual cells, it is essential to be able to derive clones from a single cell. Although this is true for established heteroploid as well as diploid cells, it is particularly applicable to primary cells. Cloning of such primary cells offers an opportunity to develop lines derived from cells which in conventional mass cultures might be overgrown by more vigorous types.

Methods for isolating a single cell for cloning include: Sanford's isolation of single cells in a capillary tube(1), implantation by Lwoff of single cells in drops of medium under mineral oil(2), trypsinization of a clone within a glass or plastic well by Puck(3), and isolation of single cells on glass squares by Schenck and Moskowitz(4).

All of these methods have been used successfully for cloning heteroploid cells with an efficiency approaching 100% in various media. With the exception of Puck's report of 25-60% efficiency for fibroblastic cells(5), however, the cloning of diploid cells is reported to be much less efficient, ranging from one clone per thousand to one clone per million cells(6). It is perhaps for this reason that certain workers clone diploid cells by diluting to a point where a single clone grows (6). Under these conditions it is apparent that a high degree of selection has been applied to the original population.

A simplified method is presented herein for the cloning of heteroploid and diploid cells. The following points are covered:

1. Procedures which help assure that the clones are derived from single cells. 2. A modified Schenck method for planting cells on small pieces of broken cover slips which facilitates their detection and isolation. 3. A description of media and conditions which provide average plating efficiencies from 5% to 50% for the diploid strains and from 5% to 100% for the heteroploid strains studied. 4. A tabulation of cloning efficiency as a function of population. 5. A tabulation of cell line derivation efficiency as a function of population.

Materials and methods. Media. All cloning was carried out in a medium consisting of Eagle's Basal Medium in Earle's Balanced Salt Solution, supplemented with 2 mM glutamine, 0.1 mM non-essential amino acids, 1 mM pyruvate and 10% dialyzed calf serum or 10% whole fetal calf serum. All media were obtained from Microbiological Associates and had been certified by their Quality Control Department as having 90% cloning efficiency for HeLa S3 cells.

* Supported in part by contract with Laboratory of Infectious Dis., Nat. Inst. of Allergy and Infect. Dis., N. I. H.