caused by chemical carcinogens in prostatic and tracheal epithelium (*16*). Whether retinoids can directly modify the development of preneoplastic lesions in bladder epithelium in vivo is not known; nor is it known whether 13-*cis*-retinoic acid has an effect on endocrine or immune mechanisms that might modulate bladder carcinogenesis. However, the recent development of an organ culture system in which both normal and carcinogen-treated bladder epithelium can be maintained in vitro (*17*) should further our understanding of the mechanism or mechanisms whereby 13-*cis*-retinoic acid inhibits bladder carcinogenesis.

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Liposome-Cell Interaction: Transfer and Intracellular Release of a Trapped Fluorescent Marker

Abstract. *When small, unilamellar lipid vesicles containing a high concentration of the fluorescent dye 6-carboxyfluorescein are incubated with either frog retinas or human lymphocytes, fluorescence distributes widely throughout each cell. Since "self-quenching" largely prevents the dye from fluorescing as long as it remains sequestered in vesicles, it is clear that a considerable amount of dye is released from the vesicles and diluted into the much larger volume of the cell.*

In recent years liposomes have been considered as vehicles for introducing membrane-impermeant substances into cells, either by membrane fusion or by phagocytosis. The list of substances whose injection has been attempted includes antitumor drugs (*1*, *2*), chelating agents (*3*), enzymes (*4*), hormones (*5*), cyclic nucleotides (*6*), and polynucleotides (*7*). Some of these studies have been done with cell suspensions or with tissue cultures, others with isolated tissues or whole animals, including human subjects (*2*). However, basic uncertainties remain about the mechanisms of interaction between liposomes and cells, in particular about the intracellular fate of incorporated material (*8*, *9*). In one study cyclic adenosine monophosphate trapped in small, unilamellar liposomes (vesicles) was reported to slow the growth of tissue culture cells (*6*). Some nucleotide was evidently released into the cytoplasmic space, but its distribution there was not determined directly, and the amount remaining sequestered in cell-associated vesicles could not be ascertained. Clearly a rapid and simple method is needed to determine the distribution in cells of substances whose injection from vesicles is being attempted. We now report results of a sensitive fluorescence technique that distinguishes between material still remaining in vesicles and that released intracellularly. The intracellular distribution of released material is determined by conventional fluorescence microscopy.

Small vesicles containing the water-soluble fluorophore 6-carboxyfluorescein (6-CF) were prepared either by sonication of a phospholipid suspension (*10*) or by injection of an ethanolic solution of lipid into an aqueous solution (*11*). The two approaches gave qualitatively similar results in studies of incorporation by cells. In the most frequently used protocol 25 mg of either dioleoyl (DOL) or dipalmitoyl (DPL) lecithin (Applied Science) was hydrated with 4 ml of a 200 m*M* aqueous solution (*p*H 7.4) of recrystallized 6-CF (Eastman). The suspension was mixed by vortexing for several minutes, then sonicated for approximately ten times the period required for optical clarification (*12*). Free 6-CF was removed by passing the sonicate through a short column (9 by 200 mm) of Sephadex G-25 at 5°C, with 135 m*M* NaCl (buffered to *p*H 7.4 with 10 m*M* Hepes) as eluant. Vesicles emerged in the void volume, whereas free dye was retarded by the gel. Lipid purity was assessed by thin-layer chromatography and gas-liquid chromatography (*13*). Quasi-elastic laser light scattering on DOL vesicles (*14*) showed more than 95 percent of the lipid in the vesicle suspension to be in the form of particles 250 to 350 Å in diameter (presumably unilamellar vesicles), and calculations based on the ratio of phospholipid to 6-CF gave an average value of about 250 Å for the vesicle diameter (*15*), assuming that the vesicles did indeed contain dye at the original 200 m*M* concentration. 6-Carboxyfluorescein closely resembles fluorescein itself in spectral properties (excitation maximum, 490 nm; emission maximum, 520 nm) but was chosen for these studies because it is more polar than fluorescein and leaks out of vesicles more slowly (*16*).

A vital phenomenon for our purposes is the "self-quenching" commonly seen with fluorescent systems. In dilute solutions of 6-CF the fluorescence is proportional to the number of dye molecules present, but as concentrations are raised above about 10 m*M* the yield per molecule drops off rapidly because of interaction between fluorophore molecules. Thus, a suspension of vesicles containing 200 m*M* 6-CF fluoresces only slightly, but the fluorescence increases more than 30-fold when dye is released (for example, by detergent) and diluted into the entire solution volume. Self-quenching thus allows material remaining in vesicles to be distinguished from that released and diluted into the much larger volume of a cell. To appreciate the magnitude of the dilution factor, consider that the internal volume of a vesicle 300 Å in diameter is about 4×10^{-18} cm^3 and that of a lymphocyte is about 2×10^{-10} cm^3 (*17*). Release into a lymphocyte of the contents of one ves-

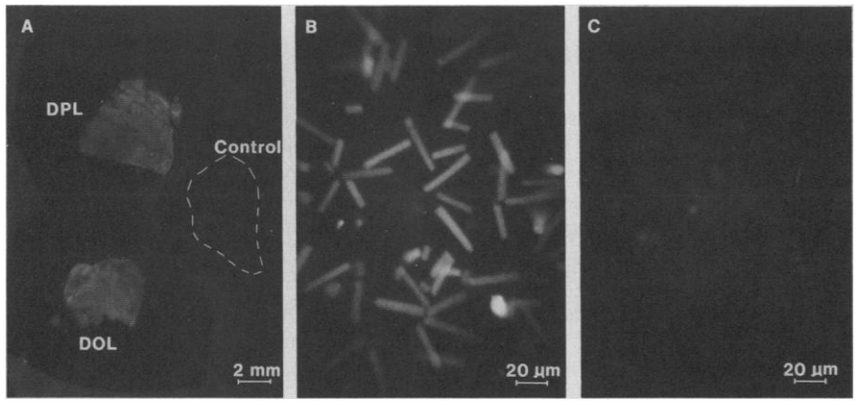


Fig. 1. Fluorescence of whole living frog retinas (A) after incubation for 3 hours at 25°C with DPL and DOL vesicles containing 100 m*M* 6-CF. The control retina, showing little fluorescence, was incubated with 10 μ*M* free 6-CF in the presence of empty vesicles. Rod outer segments isolated from the retina incubated with DPL vesicles (B) showed considerable evenly distributed fluorescence when viewed in an image intensifier. Rod outer segments from the control retina (C) were almost nonfluorescent. The incubation solutions contained a total of about 60 μ*M* vesicle 6-CF.

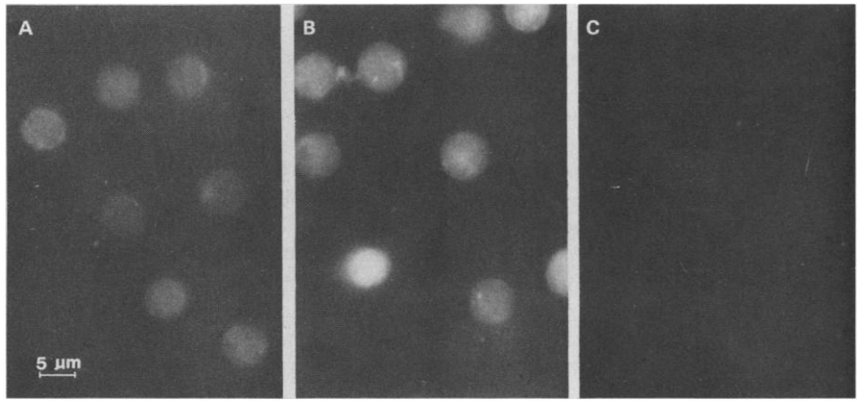


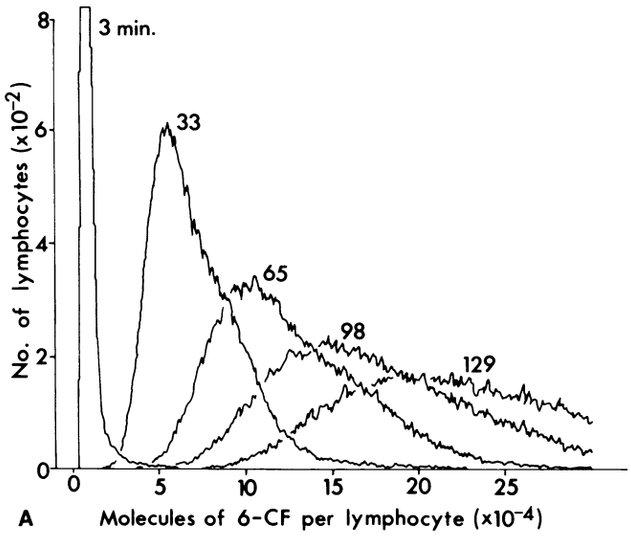
Fig. 2. Fluorescence micrographs of human peripheral blood lymphocytes incubated for 30 minutes with DPL vesicles containing 200 m*M* 6-CF at 25°C (A) and at 37°C (B). The incubation media contained 8.4 μ*M* vesicle 6-CF and 5×10^6 cells in 1 ml. Very little intracellular fluorescence appeared in a control incubation at 37°C (C) with 5 μ*M* 6-CF in the absence of vesicles.

icle containing 200 m*M* 6-CF would yield an average intracellular concentration of 4 n*M*.

Figure 1A shows live frog retinas (*18*) incubated with vesicles or free 6-CF for 3 hours, then washed extensively. The retinas incubated with DPL or DOL vesicles became highly fluorescent, all cells in the tissue taking up dye. In contrast, there was very little uptake from a control solution containing empty vesicles and free 6-CF (at a concentration greater than that which leaked out of the vesicles during the other incubations). Rod outer segments isolated from such retinas by shaking looked evenly fluorescent throughout their volumes (Fig. 1B) after incubation with vesicles containing 6-CF.

Fluorescence microscopy of lymphocytes (*19*) incubated with 6-CF vesicles (Fig. 2, A and B) showed a generalized distribution of dye fluorescence, almost certainly indicating release into the cytoplasmic space. Some cells also showed localized accumulations, and there were moderate differences in fluorescence intensity from cell to cell within a population. Mouse peritoneal macrophages took up dye much more unevenly than did lymphocytes, with numerous intensely fluorescent local accretions that may have represented phagocytic vacuoles or lysosomes.

We examined the vesicle-treated lymphocytes in a fluorescence-activated cell sorter (FACS). In the FACS (Becton-Dickinson) cells are passed in a rapidly flowing stream through a laser beam, and the amount of fluorescence (or light scattering) associated with each individual cell registers in a pulse-height analyzer.



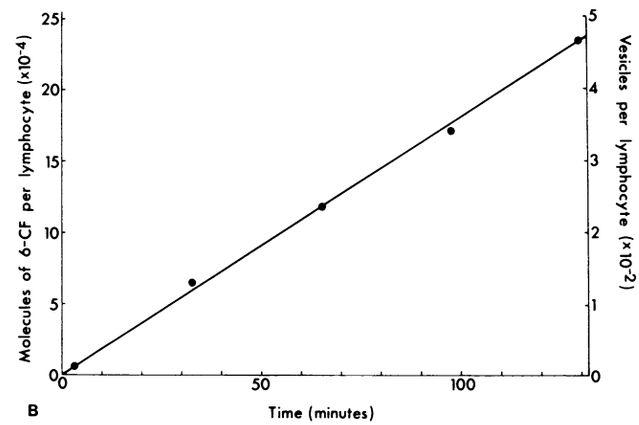


Fig. 3. Kinetics of fluorescence increase in human peripheral blood lymphocytes during incubation at 25°C with DPL vesicles containing 200 m*M* 6-CF. Histograms of cell fluorescence (A) obtained from the FACS showed linear increase of mean fluorescence (B) at a rate of 1800 ± 20 (standard error) molecules of 6-CF per cell per minute. The effective intracellular *p*H was taken to be 6.8 (*22*). The incubation medium contained 530 μ*M* vesicle lipid, 26 μ*M* vesicle 6-CF, and 4×10^6 cells in 1 ml. Spontaneous leakage of 6-CF from vesicles during the incubations (determined from fluorescence measurements before and after addition of Triton) amounted to less than 10 percent of the total trapped dye.

The result is a histogram giving the distribution of total fluorescence of individual cells within the lymphocyte population (*20*). Lymphocytes examined in the FACS after various times of incubation with 6-CF vesicles at 23°C showed a linear increase in average fluorescence up to at least 2 hours of incubation time (Fig. 3). The fluorescence after 2 hours corresponded to a mean intracellular concentration of 1.8 μM, that is, to the total release of 6-CF from about 440 vesicles per cell. Neither trypan-blue staining nor the light-scattering profile seen with the FACS showed any effect of vesicles on cell viability. Fluorescence yields corresponding to more than 3000 vesicles per cell have since been obtained with higher concentrations of DOL vesicles at 37°C. When sodium azide (an inhibitor of respiration), 2-deoxyglucose (an inhibitor of glycolysis), or both in combination were added to the incubation medium with DOL vesicles, incorporation of 6-CF was reduced by only 15 to 25 percent (*21*). This observation indicates that most of the 6-CF uptake was passive, probably by fusion of vesicles with the plasma membrane.

When we added Triton X-100 detergent to the vesicle-incubated lymphocyte suspensions (to a final concentration of 0.1 percent), 6-CF was quickly and quantitatively released from both cells and vesicles. Triton increased the fluorescence by about 50 percent in the case of cells treated with DOL vesicles and several hundred percent in the case of cells treated with DPL vesicles (*22*). Even after four or five careful washings, the lymphocytes incubated with DPL vesicles continued to shed significant amounts of Triton-releasable fluorophore (that is, vesicles) into the medium, suggesting that such vesicles do in fact bind to the cell membranes (*23*). We know, however, that most of the fluorescence observed with the FACS was not due to the small residual fluorescence yield of self-quenched 6-CF remaining in vesicles on or within the cell; if it had been, Triton treatment would have resulted in a much larger increase than actually observed and the cells would have shown fluorescent rims on microscopy (*24*).

Fluorescent solutes offer several advantages in the study of liposome-cell interaction: (i) the morphology of incorporation can be observed (at the light microscopic level of resolution); (ii) release of material within the cell can be distinguished from contamination with intact vesicles; (iii) the fluorescence-activated cell sorter can be used to determine population statistics of the process and to separate cells on the basis of their response or their state of health; and (iv) the integrity of the vesicle preparation can be monitored continuously during handling simply by observing the fluorescence of the suspension. This approach differs substantially from most radioactive tracer methods in that the fluorescent probe is sensitive to environment and to chemical transformation—either an advantage or a disadvantage, depending on the specific issue to be explored. In the present study sensitivity to other nearby 6-CF molecules is a major advantage. The use of 6-CF to monitor transfer of calcium buffers from vesicles to retinal rods is described in a separate report (*25*).

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12. The suspension was sonicated under nitrogen at 45°C for 1 hour with the microtip of a Branson sonicator (W-350) at a nominal output of 20 watts.
13. The lecithins were shipped from Applied Science Laboratories in Dry Ice and stored at −20°C under nitrogen at all times. They regularly gave only one spot on thin-layer chromatography with application of 25 μg, but sometimes showed faint secondary traces consistent with lysolecithin on application of 100 μg. There was no obvious increase in impurity after sonication. Gas chromatography on one sample of each type of lipid showed greater than 99 percent fatty acid chain uniformity. Before use of the lipid, most of the solvent was blown off with nitrogen and the residual removed in a freeze-dryer overnight.
14. Quasi-elastic light scattering was performed in collaboration with R. Nossal, Division of Computer Research and Technology, National Institutes of Health.
15. Vesicle parameters were calculated using the reasonable values 50 Å for bilayer thickness and 65 Å^2 for area per lecithin molecule. The number of lipid molecules per vesicle was estimated as $2\pi(D - 50)^2/65$, where D is the diameter in angstroms. Phosphorus analyses were done on four different sonicated vesicle preparations, and the average vesicle diameter calculated from lipid/6-CF ratios came out to 242 ± 12 Å (standard error). The likelihood of systematic error in this determination is such that the standard error quoted is misleadingly small. The result is, however, reasonably consistent with the light-scattering data; and, for the calculation, we will assume 300-Å vesicles (200 Å inner diameter) in this report. The results are very similar, but not identical, to those of H. Hauser, D. Oldani, and M. C. Phillips [*Biochemistry* **12**, 4507 (1973)].
16. The half-time of fluorescein leakage at 5°C was about 5 minutes; that of 6-CF was on the order of weeks.
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18. Live retinas were dissected from dark-adapted frogs under an infrared microscope, attached to washed black Millipore type HA filters by suction, and incubated in a stirred, oxygenated suspension of vesicles in a Ringer solution containing 1 mM Ca^{2+}, 500 mM Mg^{2+}, and 2 mM glucose. See W. A. Hagins, R. D. Penn, S. Yoshikami, *Biophys. J.* **10**, 380 (1970).
19. Human peripheral blood lymphocytes were isolated on Ficoll-Hypaque after treatment with carbonyl iron (P. Henkart and E. Alexander, in preparation). Hanks balanced salt solution buffered to *p*H 7.4 with 10 mM Hepes was used for incubations and washing. After incubation the lymphocytes were centrifuged through a cushion of 10 percent bovine serum albumin and washed by centrifugation three more times.
20. The FACS studies were done in collaboration with S. Sharrow, Immunology Branch, National Cancer Institute. The instrument was calibrated by examination of lymphocyte suspensions in a fluorometer (Aminco-Bowman) after incubation and washing. The FACS is reviewed in L. A. Herzenberg, R. G. Sweet, L. A. Herzenberg, *Sci. Am.* **234**, 108 (March 1976).
21. Lymphocytes were preincubated at 37°C for 30 minutes with 0.1 percent sodium azide, 50 mM 2-deoxyglucose, or both before addition of 6-CF DOL vesicles to the medium. Identical results were obtained when 10 percent (by weight) phosphatidylserine was added to the vesicle-forming lipid. The concentrations of inhibitor noted are sufficient to block lymphocyte functions such as capping and cytotoxicity. Our results appear to conflict with those of Poste and Papahadjopoulos (*9*), but a direct comparison is not possible because their studies and ours differed in a number of potentially important parameters, including cell type, lipid composition of the vesicles, and conditions of incubation. It is also possible that 6-CF, a trivalent anion at *p*H 7.4, adsorbs to the vesicles to some extent and renders them negatively charged.
22. When free 6-CF was incorporated into lymphocytes, the Triton-induced increase in fluorescence was 38 percent. Our calculations of intracellular fluorophore concentration include a correction factor of 19 percent for the increase in 6-CF quantum efficiency in going from an assumed intracellular *p*H of 6.8 [see E. L. Chambers and R. Chambers, *Explorations into the Nature of the Living Cell* (Harvard Univ. Press, Cambridge, Mass., 1961), p. 168] to the *p*H of the medium, 7.4.
23. Release of dye from 200 mM 6-CF vesicles results in at least a 30-fold increase in fluores-

cence. If, for example, half of the cell fluorescence recorded before Triton treatment had been due to dye in vesicles, Triton should have increased the value by a factor of at least (0.5 × 1.19 + 0.5 × 30) ≃ 16. The number 1.19 arises from the *p*H correction described in (*22*).

24. When lymphocytes were incubated with vesicles of distearoyl lecithin (transition temperature, 56°C) containing 10 m*M* 6-CF (self-quenched by only a factor of 2), the cells developed clearly defined fluorescent rims, indicating dye in vesicles at the cell surface. Binding of vesicles to cell membranes has recently been described independently by L. Huang, K. Ozato, and R. E. Pagano [*Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35**, 1703 (1976)] and R. Martin and R. C. MacDonald [*J. Cell Biol.* **70**, 515 (1976)].
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7 September 1976

Hyperosmolality in Intraluminal Fluids from Hamster Testis and Epididymis: A Micropuncture Study

Abstract. *Intraluminal fluids from the hamster seminiferous tubules, rete testis, and caput, corpus, and cauda epididymidis were obtained in vivo by micropuncture. Rete testis fluid is isosmolar with serum. Fluids from the seminiferous tubules and all sites in the epididymis are hyperosmolar.*

Spermatozoa are neither motile nor fertile immediately after their release from the germinal epithelium. By the time they reach the cauda epididymidis, they have acquired the capacity for fertility and motility. Studies of sperm held by ligation in the proximal epididymis (*1, 2*) have shown that this maturation is not the result of aging alone, but is partially dependent on exposure to the epididymal milieu. It seems likely that maturation has some correlation with the progressive changes that occur in the ionic and organic constituents of the tubule fluids along the length of the excurrent ducts (*3*). However, knowledge of the composition of these fluids is limited. One basic factor which has received only minimal attention is their osmolality.

For osmolality studies in the boar (*4*), ram (*5, 6*), and hamster (*6*), testes and epididymides were obtained at slaughter, and the contents squeezed out from cut tubules. The development of a technique for cannulation of the rete testis of the conscious ram (*7*) marked the first time that fluid had been collected from the male reproductive tract in vivo. Later, micropuncture techniques made it possible to obtain fluid directly from the seminiferous tubules and proximal ductus epididymidis in vivo (*8, 9*). Here we report the results of the determination of osmolality in fluids obtained by micropuncture from the seminiferous tubules, rete testis, and five regions of the epididymis of the golden hamster, *Mesocricetus auratus*.

Adult male golden hamsters (100 to 170 g; Lakeview Hamster Colony) were anesthetized and prepared for micropuncture as described previously (*10, 11*). Samples of rete testis fluid were collected 2 to 3 hours after ligation of the efferent ducts. All samples were sandwiched between columns of water-equilibrated mineral oil in the collection pipettes to prevent evaporation, and were centrifuged at 13,460*g* for 15 minutes to separate the spermatozoa. Blood was collected from each animal by cardiac puncture at the end of the experiment.

Osmolality was determined on duplicate portions of each sample by measuring freezing-point depression with a nanoliter osmometer (Clifton Technical Physics). A special sample holder was constructed for the osmometer with sample wells that extend below the surface of the cooling module; the wells were thus in line with the cooling waves generated by the stage. To ensure proper thermal maintenance of all samples, a compound (Dow Corning 340 silicone) that would retard heat transfer was used as an interface between the sample holder and the cooling module. By means of a vertical transfer apparatus (Bunton Instrument) and constriction pipettes with 60 μm tips, 5- to 7-nl volumes were transferred to the osmometer from the samples, which were either sandwiched between columns of oil in the collection pipettes or deposited under oil in glass sample dishes. A standard curve was run with each group of three samples. Samples were either analyzed on the day of the experiment or stored under oil at −20°C and analyzed within 3 days. Storage for 1 to 3 days did not alter the osmolality.

The osmolalities in the seminiferous tubules (384.1 milliosmoles per kilogram) and all regions of the epididymis studied were significantly higher ($P < .01$) than the serum osmolality (Fig. 1). Rete testis fluid osmolality was not different from serum ($P < .05$). The osmolality decreased progressively down the length of the ductus epididymidis from 417.0 and 408.4 mosmole/kg in the caput and corpus, respectively, to 358.5 mosmole/kg in the proximal cauda ($P < .01$), then to 339.7 and 331.6 mosmole/kg in the distal cauda and epididymal vas, respectively ($P < .05$).

To determine whether the hyperosmolality of our samples was due to enzymatic degradation of the constituents of the fluids after they were collected, several proximal cauda samples were centrifuged in a cold room immediately after collection and held on ice until their transfer to the osmometer. The analysis was completed within 3 hours after collection. No significant difference was observed between the value (± standard error) of 340.4 ± 9.0 mosmole/kg ($n = 7$) for the cold samples and the original value of 358.5 ± 7.4 mosmole/kg ($n = 21$) for samples centrifuged and held at room temperature.

Additional experiments were performed to determine whether the samples were losing water into the surrounding oil. A small volume of tritiated water was injected into a segment of the ductus epididymidis in the caput and cauda. Then, a sample was withdrawn from the same location by micropuncture, centrifuged, and either left in the collection pipette or deposited under oil in a sample dish. In both cases, duplicate portions of the tubule fluid and of the oil near the fluid-oil interface were transferred to glass vials for liquid scintillation counting. No significant difference from background was ever observed for any of the oil samples, while there were as many as 20,000 count/min in a comparable portion of tubule fluid.

Hyperosmolality in the fluids of the male reproductive tract has been mentioned but not emphasized or statistically documented in previous literature. Several investigators reported that the fluids they collected from the testis and epididymis of large slaughtered animals were hyperosmolar, although the samples may have been contaminated or have undergone postmortem changes. Salisbury and Cragle (*12*) found that the osmolalities in the testis and cauda epididymidis of the bull tended to be higher than those in both blood and semen. Similarly, Scott *et al.* (*5*) reported that the osmolalities in the testis, caput and cauda epididymidis, and vas deferens of the ram were hyper-