polysaccharide, but not with its presence in a cell wall protein or cytoplasmic residue. Methyl groups may be esterified to the hemicellulosic matrix of the primary wall which, in the case of *Micrasterias*, is rich in uronic acids (*11*).

Autoradiography therefore reveals two patterns of incorporation of material into cell wall which are spatially and biochemically distinct. Comparing these with Robertson's two hypothetical processes, extension and hardening, we could conclude that methionine incorporation is associated with wall extension and glucose incorporation (microfibril synthesis) with hardening. The participation of microfibril synthesis in hardening of plastic wall is not surprising since Robertson has argued that this is the case in hyphal growth (*5*). Nor is it difficult to envisage a role for microfibrils in a process that probably involves stiffening and reinforcement. The relation between methylation and Robertson's extension process is not so easily dealt with. Extension probably depends on wall loosening and some wall synthesis, although a precise understanding of the extension process in tip growth and in other types of cell wall growth has been elusive (*12, 13*). There is no simple causal relation between methylation of existing wall and extension, although inhibition of methylation may reduce cell elongation in some cases (*14*). It may be, in *Micrasterias*, that methylation accompanies the incorporation of new matrix into wall, and that matrix incorporation is linked to wall extension. Physical properties of the wall do depend upon the amount and kind of matrix present (*15*), and during growth matrix incorporation could act to force microfibrils apart or serve as a lubricant to facilitate microfibril slippage (*13, 16*). If extension were more directly the result of enzymatic hydrolysis or the breaking of acid-labile bonds, matrix incorporation must in any case accompany these changes in order that the original wall thickness and composition be maintained in regions of extension. Methylation, matrix incorporation, and wall extension are probably interrelated in some fashion. Tip growth in *Micrasterias* may provide an additional opportunity for study of this interrelation.

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7. Cells normally grown in Waris medium were allowed to develop for from 5 to 90 minutes in Waris medium containing [1-^{3}H]glucose or [methyl-^{3}H]methionine (200 μc/ml). Cells were washed twice with fresh medium and lysed with an ultrasonic cleaner (20 khz), with the time through washings to lysis standardized at 5 minutes. Primary walls were picked out by hand and mounted on slides for standard autoradiographic processing with the use of Ilford L-4 emulsion.
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17. Supported by the National Research Council of Canada. We thank I. E. P. Taylor for his interest and comments.

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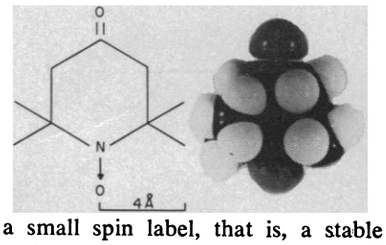
Viscosity of Cellular Protoplasm

Abstract. *The protoplasmic viscosity was studied by using a small spin label having high permeability and broad solubility properties and nickel chloride as an extracellular spin-subtracting agent to localize signal inside cells. The viscosity is variable and in some cells is many times that of water or phospholipids, suggesting that lateral diffusion in biological membranes is important to cell function.*

The internal viscosity of cells is undoubtedly important to many cellular processes and cells might be expected to have considerable diversity in internal viscosity based on size differences and amount of internal membrane structure. Several approaches have been used in attempts to determine intracellular viscosity. In one study, centrifugation coupled with microscopy was used to observe the rate at which intracellular organelles diffuse through the cytoplasm. For eggs of the sea clam *Spisula*, the protoplasmic viscosity was estimated to be 4.3 centipoise (cp) (*1*). Another method involved the incorporation of magnetic particles into cells and subsequent observation of the viscous drag they experience in response to an external magnetic field. The internal viscosity of the giant amoeba *Chaos chaos* was estimated to be 3 to 11 cp by this method (*2*). In a detailed study of the interior of cultured chicken cells, Crick and Hughes (*3*) found a somewhat more rigid protoplasm with evidence of thixotropy, and suggested a value for the modulus of rigidity of about 10^2 dyne/cm^2. The magnetic particle method was also used in these experiments. More recently, nuclear magnetic resonance experiments employing the spin-echo technique were carried out to make approximations of the self-diffusion coefficient of water protons. In yeast, the diffusion coefficient was about 3.4 times less than in water (*4*). With the exception of this last study, all reports have dealt with a bulk or macroviscosity. It is not obvious nor perhaps expected that the microviscosity relevant to the diffusion and rotation of small molecules would mimic the bulk viscosity of a solution or of protoplasm.

Tempone (2,2,6,6-tetramethylpiperidone-*N*-oxyl radical), whose structure is shown below,



is a small spin label, that is, a stable organic radical with an unpaired electron. We have measured the molecular motion of tempone in several different types of cells (*5*). The spin label was allowed to passively diffuse into cells, and spin label signal originating from outside the cell boundaries was eliminated by having 0.5*M* $NiCl_2$ in the medium (*6*). Nickel is paramagnetic yet gives no visible electron spin resonance (ESR) signal in liquid medium

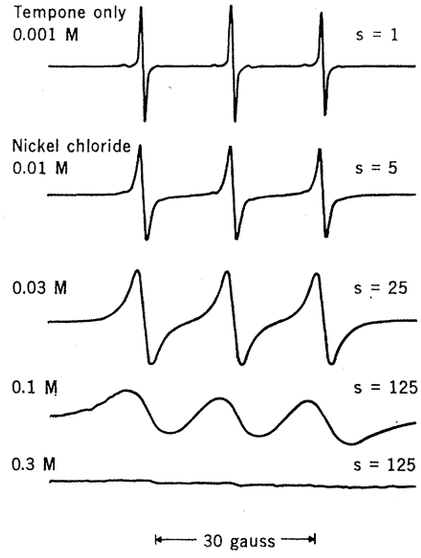


Fig. 1. Effect of $NiCl_2$ on the ESR signal of tempone at room temperature. The spectrum at the top is for $10^{-3}M$ tempone in water; the next four spectra show the effect of sequentially increasing $NiCl_2$ concentrations. At the right of each spectrum the spectrometer sensitivity (s) is indicated. All measurements were made on a JEOL X-band spectrometer, model JES-ME-1X, equipped with a variable temperature unit constructed in our laboratory and having an estimated accuracy of $\pm$ 0.1°C.

in the biological temperature range. Figure 1 shows ESR signals taken of $10^{-3}M$ tempone dissolved in water with increasing amounts of $NiCl_2$. The paramagnetic nickel at high concentrations broadens the tempone signal beyond detection; the basis for this broadening by magnetic dipole interactions has been well studied (*7*). Our ESR measurements (*6*) and additional experiments with the radioactive isotope ^{63}Ni show that no detectable amount of nickel enters or becomes associated with the cells (*8*).

For comparative purposes, the relation between viscosity (η) of glycerol-water mixtures and the measured rotational correlation times (*9*) (τ_c) of tempone in these mixtures was studied. A log-log plot of these quantities is shown in Fig. 2A. The slope of this relation would be unity if microviscosity agreed with macroviscosity so that Stokes' equation $\tau_c = 4\pi\eta a^3/3kT$ was completely valid. (In this equation, a, k, and T are the particle radius, Boltzmann's constant, and absolute temperature, respectively.) It might be expected that some long-range order exists in most solutions, particularly those containing anisotropic and noninert molecules. This could lead to a nonlinear relation between η and τ_c, and it seems reasonable that τ_c rather than η gives the most appropriate "viscosity" for the rotation and diffusion of small molecules.

Figure 2B shows τ_c values plotted against temperature for six different cells: two bacteria, a yeast, a green alga, a higher plant, and a mammalian cell. Values of τ_c for tempone in water are also given and serve as a comparative base for the cells. Tempone has both hydrocarbon and polar contributions in its structure and partitions between equal volumes of methyl oleate and water with a partition coefficient of about 2, favored to the methyl oleate phase. Inasmuch as the cell interior is mostly composed of water, the spin label signals observed should reflect mainly the viscosity of the aqueous protoplasm. This viscosity may be significantly increased by the long-range ordering effects of internal membrane networks and other hydrocarbon-rich zones. The cells with the largest values of τ_c (and therefore the largest microviscosity) in Fig. 2B are expected to have extensive internal membrane structures. The properties of cells conferring high microviscosity to the protoplasm are probably not due to high solute concentrations since tempone in $1.6M$ sucrose has $\tau_c = 0.42 \times 10^{-10}$ second.

An additional consequence to be expected for a more ordered aqueous cell interior would be a reduced hyperfine coupling constant (A_N) for the spin label. The value of A_N for a particular spin label is smaller in solutions of lower dielectric constant, and the structuring of water would likely reduce its ability to stabilize the charged nitroxide structure (*10*). We found that A_N decreased slightly as τ_c increased with the different cells, in agreement with these considerations.

The degree to which the aqueous portion of the cell is probed by tempone is worth some emphasis. Lyophilized samples of the spin-labeled cells yielded τ_c values ranging from 1.5×10^{-10} to 4.8×10^{-10} second (*11*) and showed considerable reduction in A_N compared to the native samples. For example, lyophilized yeast at 15°C had $A_N = 14.7$ gauss compared to 16.1 gauss for the native sample. Tempone has $A_N = 14.5$ gauss in methyl oleate and 16.3 gauss in water. Comparison of these values indicates that the spin label is in an aqueous environment in the native cells and that significant contributions from hydrocarbon zones appear only after the water has been removed.

It is likely of some importance that the values of τ_c for tempone in the

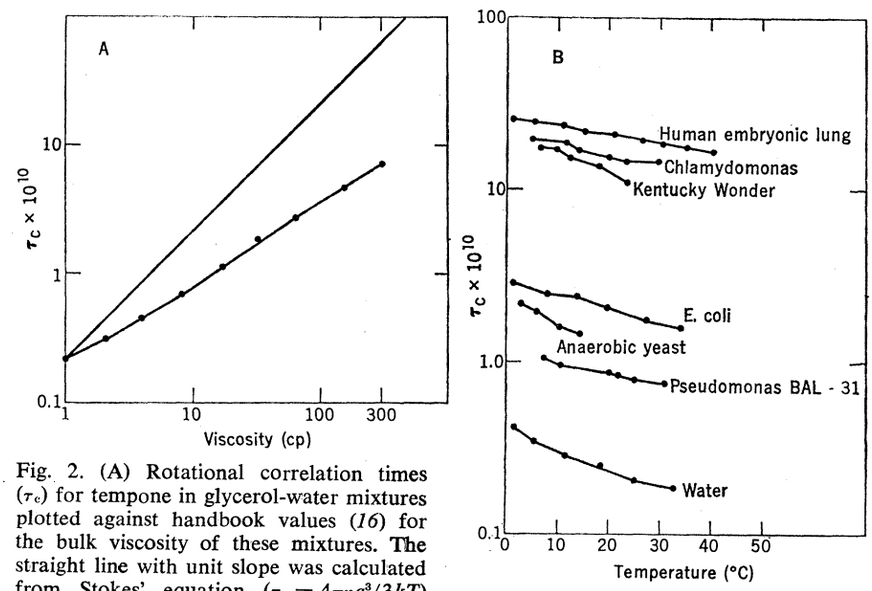


Fig. 2. (A) Rotational correlation times (τ_c) for tempone in glycerol-water mixtures plotted against handbook values (*16*) for the bulk viscosity of these mixtures. The straight line with unit slope was calculated from Stokes' equation ($\tau_c = 4\pi\eta a^3/3kT$) with $a = 3$ Å, $\eta = 1$ cp, and $T = 20$°C. (B) Values of τ_c for tempone in various cells plotted against temperature. Human embryonic lung cells were a gift from S. Person; Kentucky Wonder pole bean root tips were a gift from J. Lyons; and *Chlamydomonas* cells were provided by L. Adams. Cell suspensions were treated with $0.5M$ $NiCl_2$, $0.001M$ tempone, and in some cases $10^{-5}M$ $K_3Fe(CN)_6$ to prevent spin reduction. After 5 to 15 minutes the cells were centrifuged, resuspended in a small volume of the same solution, and placed in capillaries (inside diameter 0.9 mm) for ESR measurements. Root tips were soaked for 3 hours in $10^{-3}M$ tempone, and $0.5M$ $NiCl_2$ was added before the ESR measurements.

protoplasm of the mammalian, plant, and algal cells are far greater than those in lyophilized samples or in isolated phospholipids. In these three cells, tempone experiences much greater freedom of molecular motion in cellular hydrophobic zones than in cellular aqueous zones. These data suggest that, in cells where internal membrane structure abounds, the aqueous regions have such a high microviscosity that diffusion along the plane of the membrane or within the membrane structure may be faster and more efficient than through the three-dimensional aqueous protoplasm. Our observations indicate that the lateral translational diffusion in membranes reported by Frye and Edidin (*12*), Träuble and Sackmann (*13*), Devaux and McConnell (*14*), and Scandella *et al.* (*15*) may be extremely important to vital cellular logistic processes.

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9. Values were calculated from $\tau_c = K(W_{-1} - W_1)$, simplified to use line height (h) ratios, where $W_{-1} - W_1 = W_1[(h_1/h_{-1})^{1/2} - 1]$. This equation, or modifications of it, has been treated elsewhere both theoretically [D. Kivelson, *J. Chem. Phys.* **33**, 1094 (1960)] and in application to membranes [A. Keith, G. Bulfield, W. Snipes, *Biophys. J.* **10**, 618 (1970)].
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Cell Culture of Mammalian Endometrium and Synthesis of Blastokinin in vitro

Abstract. *Endometrial cells obtained from mature female estrous rabbits can be grown in cell culture with the aid of insulin. These cells, after several days in culture, are capable of synthesizing blastokinin by induction with progesterone for 48 hours.*

Recent evidence on the action of steroids on target cells of chick oviduct (*1*), mammary explants (*2*), and ovarian follicular cells in vitro (*3*), indicates that the interaction of hormones is required for cell proliferation and subsequent cell differentiation. Previous workers in attempts at in vitro cultivation of mammalian endometrium failed to take this into consideration (*4*), which probably accounts for their inability to maintain long-term viable tissue cultures that respond to steroid hormones. It had been observed, however, that a cell line from human endometrial adenocarcinoma has recently been established without exogenous addition of steroids or other hormones (*5*). On the other hand, survival of short-term human endometrial tissue in organ culture increased when insulin was added to the culture prior to stimulation by progesterone (*6*). Studies on chick oviduct epithelium also indicated that estrogen is necessary for cell division and that progesterone added to cells previously stimulated by estrogen blocked cell proliferation.

These results and those from mammary explant cultures (*2*) suggested to us the possibility that tissue cultures of rabbit endometrium could be established if insulin alone or in combination with estrogen was added to the medium prior to administration of progesterone. In vitro cultures of rabbit endometrium would serve as an excellent model system for testing the interaction of various hormones, since it has been shown in vivo that rabbit endometrium secretes "blastokinin" in the progestational state from days 3 to 9 of pregnancy (*7*). Blastokinin has been found to be an inducer of blastocyst growth in vitro (*7*), stimulates mitotic activity in cells of diapausing blastocysts (*8*), and stimulates RNA and protein synthesis in rabbit blastocysts (*9*). Blastokinin is synthesized in uteri of ovariectomized rabbits by exogenous addition of progesterone (*10*). Therefore, we have developed a cell culture system to investigate the control and regulation of hormones on the production of blastokinin. Monolayers (mixed culture) of uterine endometrium have been established in our laboratory both as primary and secondary tissue cultures which proliferate under the influence of insulin, and primary cultures secrete blastokinin after stimulation with progesterone.

Primary tissue cultures of rabbit endometrium were established from uterine tissues obtained from mature estrous New Zealand white female rabbits with no previous history of matings. Whole uteri were removed surgically from rabbits, washed several times in 0.9 percent sterile saline, and exposed to ultraviolet light in a sterile transfer hood for 10 to 15 minutes to ensure sterile organs. With the ultraviolet off, uteri were cut open and the uterine epithelium was carefully removed with forceps and curved surgical scissors. Endometrium was finely minced, with curved scissors, in sterile saline (0.9 percent) and placed in sterile, 40-ml capacity, screw-cap centrifuge tubes containing four to five volumes of 0.25 percent sterile trypsin. Tissues were agitated gently by hand for 20 to 30 minutes at room temperature and centrifuged. The trypsin solution was decanted and cells were suspended in four to five volumes of medium 199 containing Hanks basal salts and 10 percent fetal calf serum (Colorado Serum Co.).

Cells were placed down (2×10^3 to 5×10^3 cells per milliliter) in 60-mm sterile Falcon plastic petri dishes containing 3 to 5 ml of medium 199 with Hanks base containing 10 percent fetal calf serum, antibiotic-antimycotic mixture (Grand Island Biological Co.), and insulin (bovine pancreas, Calbiochem),