−30 mv) and did not produce action potentials in response to electrical depolarization or application of acetylcholine (*16*). Similar studies on adult gerbil and human adrenal medullary cells in our laboratory, however, indicate that these cells can have resting potentials of at least −55 mv, and that they are capable of generating overshooting action potentials (*17*). If action potentials do prove to mediate some forms of endocrine secretion, it will be of interest to see whether the phenomenon is confined to cells of neural crest origin.

Many specific physiological considerations such as the ionic mechanism of action potentials, the relation between excitation and secretion, and receptor sensitivity could not be adequately studied in our human tumor systems because of the small amounts of tissue available in surgical specimens. We have, therefore, extended our investigations to reported animal models of APUD tumors (*18*). We have studied rat pheochromocytoma and bovine MCT cells, and have observed that these cells, like their human counterparts, are electrically excitable, and that the pheochromocytoma cells grow processes in response to NGF (*19*). These animal tumors might therefore be useful models for expanded studies.

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3. Tissue from five human pheochromocytomas obtained at surgery was minced, dissociated in 0.25 percent trypsin in Ca- and Mg-free Earle's salt solution for 30 to 45 minutes, suspended in McCoy's 5A medium with 20 percent fetal calf serum (FCS) or Dulbecco's modified Eagle's medium with 15 percent FCS, and grown on 35-mm plastic Falcon tissue culture dishes with inocula of either 5,000 or 50,000 viable cells per dish. Additional minced tissue from one of the tumors was mechanically dissociated by trituration in a Pasteur pipette. Cultures were incubated at 37°C in a mixture of 5 percent CO_2 and 95 percent air. Medium was changed every 3 to 5 days. Five biological units (BU's) of 2.5*S* mouse salivary gland nerve growth factor (NGF) was added to some cultures from each tumor. Histologic sections from areas of the tumors used for cell cultures showed no normal adrenal tissue or neurons.
4. Cells from two human MCT's were mechanically dissociated and grown on Falcon tissue culture dishes in McCoy's 5A medium with 20 percent FCS, sometimes supplemented with 5 mg of L-thyroxine or 5 BU's of NGF, or both, per milliliter.
5. Cells from two human BC's were mechanically dissociated and plated in McCoy's 5A medium with 20 percent FCS, sometimes supplemented with 5 BU of NGF per milliliter either directly on Falcon tissue culture dishes, or on monolayers of fibroblasts whose growth had been arrested with cytosine arabinoside.
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Nuclear Magnetic Resonance Patterns of Intracellular Water as a Function of HeLa Cell Cycle

Abstract. *Nuclear magnetic resonance relaxation time (T_1) of the intracellular water protons and water content were measured in synchronized HeLa cells. The T_1 was maximum (1020 milliseconds) in mitotic and minimum (534 milliseconds) in S phase cells. The cyclic pattern of T_1 values correlated well with the chromosome condensation cycle. By treating cells with spermine, it was possible to alter T_1 without a significant change in the water content. The results of this study suggest that an additional variable, namely, the conformational state of macromolecules, should be included in any expression explaining the shortened relaxation times of water protons in biological systems.*

The importance of water in biological systems is obvious, since it constitutes 70 to 90 percent of the mass in most living systems. The structure and function of this simple molecule in biology, however, is not yet completely understood. One of the quantitative methods available to study the physical properties of water is nuclear magnetic resonance (NMR) spectroscopy. Primarily, NMR spectrometers are comprised of a magnet and a high-frequency radio transmitter which produce perpendicular magnetic fields. The hydrogen nuclei of water molecules will absorb energy when placed in a strong magnetic field at a specific resonance frequency. In pulsed NMR, the water hydrogen protons absorb energy during a brief (microseconds) pulse of radio-frequency ener-

gy. Following the brief pulse of high-frequency energy, the excited system relaxes back to equilibrium with certain time constants known as T_1, the spin-lattice relaxation time, and T_2, the spin-spin relaxation time. (In the present study, we confine our discussion to T_1 only.)

Since the time needed to return to equilibrium depends on the interaction of the water molecule with its environment, it is dependent upon the motional freedom available to the water molecules. Pure liquid water at 25°C has a T_1 of about 2500 msec, while crystalline ice has a T_1 on the order of 10^2 msec (*1*). It has been shown that the T_1 of water protons is shorter in living tissues than in liquid water, indicating a restricted mobility of at least a portion of the water molecules (*2*). Different tissues from the same animal may exhibit different T_1 values. For example, the T_1 values for rat brain and stomach are 600 msec and 250 msec, respectively (*3*). Cancerous tissues of humans were reported to have higher T_1 values as compared to normal tissues of the same organ (*4*). Similarly, the T_1 values of rat gastrocnemius muscle were found to decrease from 1206 msec to 723 msec during normal development (*5*). This decline is closely related to an increase in the molecular complexity of the tissue as well as a decrease in its water content.

These studies raise the important question of what is the relationship between T_1, the physiological state of the cell, and the water content. Of particular interest to us was to determine how T_1 and water content are related to the macromolecular changes associated with the specific phases of the HeLa cell cycle. To answer this question, we measured T_1 and the percent water in random and synchronized populations of HeLa cells. The results of this study indicate that there are cell cycle phase-specific changes in T_1 and water content in HeLa cells. Changes in T_1 do not always correspond to changes in cellular water content. An abstract of this study has appeared elsewhere (*6*).

HeLa cells were grown as monolayer cultures in Falcon plastic dishes at 37°C in Eagle's minimal essential medium (MEM) supplemented with Eagle's nonessential amino acids, heat-inactivated fetal calf serum (10 percent), sodium pyruvate, glutamine, and penicillin-streptomycin mixture (*7*). Cells were synchronized by different methods in the pre-DNA synthetic period (G_1); DNA synthesis period (S); post-DNA synthetic period (G_2); and in mitosis (M). Briefly, a random population of HeLa cells

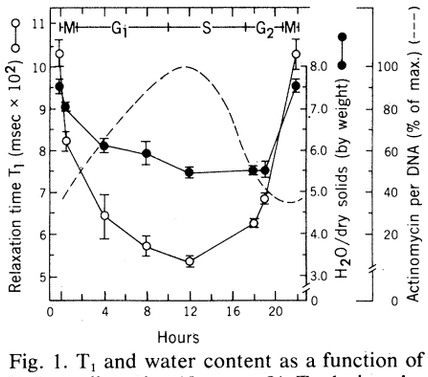

Fig. 1. T_1 and water content as a function of HeLa cell cycle. (○———○) T_1 during the cell cycle (mean of eight to ten experiments). Bars denote standard error of the mean. (●———●) Water content during the cell cycle. (— — —) Actinomycin-D binding ability of the chromatin. [Data for the dashed line are from Pederson and Robbins (*13*)]

were incubated with thymidine (2.5 m*M*) for 16 hours. Then the drug was removed by washing, and cells were reincubated in regular medium for 8 hours, at the end of which thymidine was added again to the medium. After a period of 16 hours, the second thymidine block was released to obtain a synchronous population of S phase cells. Usually, synchronized S and G_2 populations were obtained by harvesting cells at 1 hour and 6 hours, respectively, after reversal of the second thymidine block (*8*). To obtain a mitotic population, HeLa cells were first partially synchronized by a single thymidine block of about 20 hours duration. Four hours after reversal of the thymidine block, cells were incubated for about 10 hours with colcemid (0.05 mg/ml) or in a chamber filled with N_2O at a pressure of 80 lb/in.^{2} (1.36 atm). At this time, 80 to 90 percent of the cells were in mitosis. The rounded and loosely attached mitotic cells were selectively detached by gentle pipetting, which yielded a population with a mitotic index of 98 percent (*9*). In HeLa cells, mitotic block by N_2O is completely reversible, whereas colcemid block is not. Incubation of N_2O-blocked mitotic cells under regular culture conditions for 3 hours yielded a highly synchronous population of G_1 cells (*9*).

For NMR measurements, about 2×10^7 cells were required for each sample. Samples were prepared by gently scraping the cells from the culture dish with a Teflon policeman and centrifuging them in a narrow glass tube at 1000*g* for 20 minutes. The supernatant medium was removed by suction without disturbing the tightly packed cell pellet, and the samples were sealed and placed on ice until rewarmed to room temperature for NMR measurements. The NMR measurements were routinely done between 1 and 2 hours after collection to ensure as much reproducibility as possible between experiments. An independent time study showed that there was no change in the T_1 of the pelleted samples for up to 4 hours when they were kept at 4°C. In order to examine whether there were any cell cycle phase-specific differences in membrane fragility, we performed trypan blue dye-exclusion tests on pellets prepared as described above. The results of these tests showed less than 2 percent dead cells in any phase, indicating that the contribution of dead cells to the observed changes in T_1 and water content was negligible.

A Bruker SXP NMR spectrometer (30 Mhz) was used to measure the T_1 by a 180-τ-90° pulse sequence at 25°C (*10*). The water, culture media, salt, and drug solutions were tested for the presence of paramagnetic impurities by comparison to a water standard. The addition of 10 percent fetal calf serum to Eagle's MEM slightly depressed the T_1 of the medium, probably owing to the macromolecular content of the serum. All other solutions showed no depression of the T_1 of the distilled water in which they were dissolved. All cell samples were measured for water content by drying in an oven at 100°C until a constant weight was achieved.

Random populations of HeLa cells had a T_1 of 667 msec and a water content of 86.0 percent, or 6.62 g of H_2O per gram of dry solids (average values of five experiments). The method of harvesting the cells (that is, trypsinization versus scraping) had no effect on these parameters. Synchronized populations (*11*), however, demonstrated a reproducible pattern of changes in T_1 associated with the specific phases of the cell cycle (Fig. 1). Mitotic cells had the mean maximum T_1 value of 1020 msec ± 84 msec (standard deviation). The T_1 values for mitotic cells obtained by colcemid treatment were identical with those collected by N_2O block. At 30 minutes after the reversal of the N_2O block, when the damage to the mitotic spindle was repaired and the chromosomes were realigned on the metaphase plate (*12*), the T_1 had decreased rapidly to a mean value of about 800 msec. This realignment of the chromosomes and subsequent decrease in T_1 was prevented if the mitotic cells were held at 4°C immediately after the release of the N_2O block. This suggests that it is not the time interval after N_2O block, but the structural organization following the reversal of the block, that is responsible for the decrease in T_1. The

Table 1. Effect of chromatin condensation on the T_1 of synchronized S phase HeLa cells.

Treatment	Duration of incubation	Sample No.	T_1 (msec)	H_2O (%)	H_2O/dry solids (by wt.)
Control	1 hour		538	84.4	5.42
Spermine (0.02*M*)	1 hour	1	637	84.3	5.35
		2	638	84.6	5.50
Average			637.5	84.45	5.42
Control	2 hours		546	84.6	5.49
Spermine (0.02*M*)	2 hours	1	711	85.3	5.78
		2	701	85.0	5.67
		3	695	84.8	5.57
Average			701	85.0	5.67

T_1 continued to decrease throughout G_1 and reached its mean minimum value of 534 ± 43 msec in S phase. As the cells progressed through G_2, the T_1 started to increase from 621 ± 25 msec to 690 ± 4 msec and ultimately returned to the maximum as the cells reentered mitosis. The cyclic pattern of T_1 shown in Fig. 1 appears to be inversely related to the degree of chromatin condensation, as measured by the amount of actinomycin-D bound to DNA during the cell cycle (*13*). To what extent the conformational changes in chromatin during the cell cycle are responsible for the changes in T_1 remains to be elucidated.

Recently it has been proposed by some investigators that in biological samples the variation in T_1 is a simple linear function of the water content (*14*). A comprehensive study on the effects of ionic concentration on the T_1 of rat liver tissue and V79 Chinese hamster lung fibroblasts revealed that within certain limits the relationship between water content and T_1 may be linear, but in treatments with hyper- and hypotonic osmolarities of NaCl and KCl the T_1 changes in a nonlinear manner (*15*). In the present study, the graph of water content versus T_1 for synchronized HeLa cells showed that the water content of the cells was variable over the cell cycle and did not correlate in a strictly linear manner with T_1 (Fig. 2A). Furthermore, in the transition from S to G_2, significant changes in T_1 were observed independent of changes in water content (Figs. 1 and 2A).

To further test the exactness of the relationship between hydration and T_1, we incubated random populations of HeLa and Chinese hamster ovary cells in solutions of 0.03 to 0.5*M* NaCl for 15 minutes at 37°C to allow them to equilibrate with the solutions and then centrifuged them to a pellet for NMR measurements. Microscopic examination demonstrated that marked changes had occurred in the morphology of the cells, including rupture of the cell membrane of HeLa cells outside the range of 0.05 to 0.25*M* NaCl. Chinese hamster ovary cells (grown as monolayers in McCoy's modified medium) were able to withstand a greater range of salt concentration, but they too demonstrated shrunken nuclei, crenated nuclear membranes, and condensed chromatin at high salt concentrations. In hypotonic solutions of NaCl, the cells were swollen and the cytoplasm was highly vacuolated. In Fig. 2B, the nonlinearity of water content versus T_1 is shown for both the cell lines. It appears that within narrow physiological limits the changing cellular water content and T_1 are linear, but when gross morphological changes become visible, this relationship deviates from linearity.

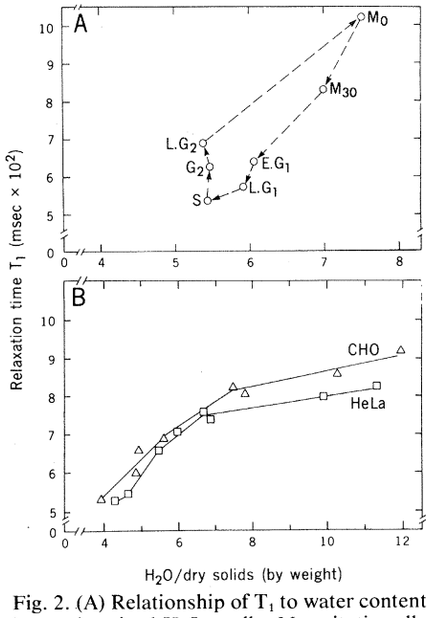

Fig. 2. (A) Relationship of T_1 to water content in synchronized HeLa cells. M_0, mitotic cells immediately after reversal of N_2O block; M_{30}, 30 minutes after reversal of N_2O block; $E.G_1$, early G_1; $L.G_1$, late G_1; S, 1 hour after reversal of a double thymidine block; G_2 and $L.G_2$, 6 and 7 hours after release of second thymidine block. Each point is the mean of eight to ten experiments. Standard errors for H_2O/dry solids (by weight) and for T_1 are the same as shown in Fig. 1. (B) Relationship of T_1 to water content in random populations of Chinese hamster ovary (*CHO*) cells (△) and HeLa cells (□) treated with various concentrations of NaCl (0.03 to 0.5*M*).

A dividing cell undergoes continuous morphological changes, which are associated with the biochemical events peculiar to each phase of the cell cycle. The interrelationship between T_1 and the cyclic pattern of cell growth and division may be influenced by changes in the state of macromolecules as well as the water content. The actinomycin-D binding ability of HeLa cells during the cell cycle (Fig. 1) gives an indication of the openness of the chromatin in each phase. Since the NMR data seemed to be related to the amount of surface area of the chromatin that was available to affect the motional freedom of water, and chromatin being a sizable fraction (30 percent) of the macromolecular component of the cell, it occurred to us that not only the amount of macromolecules but also their conformational state play an important role in the variation of T_1. It has been shown that the NMR parameters of tissues (muscle and lens) can be altered, without changes in water content, by inducing conformational changes in macromolecules by heat denaturation (*16*). Similarly, we wanted to determine how induced conformational changes in the chromatin of HeLa cells, produced by treating them with agents known to cause chromatin condensation, would affect the T_1 in a living system. For this purpose, we selected the polyamine, spermine, on the basis of published reports (*17*) which show that this compound significantly increases the induction of premature chromosome condensation in cell fusion studies. Synchronized S phase cells were incubated with and without spermine (0.02*M*) in the culture medium for 1 to 2 hours at 37°C and prepared for NMR measurements. The data in Table 1 clearly indicate that this treatment increased the T_1 without significant change in the water content (that is, the T_1 values for tissue water can be uncoupled from hydration effects). Such a result was expected on the basis of data in Fig. 1. These observations suggest that one may be able to manipulate the T_1 by altering the conformational state of chromatin. However, it is important to remember that in addition to chromatin condensation many other changes, such as exchange of macromolecules and ions between the nucleus and cytoplasm as well as between the cell and its external environment, may be taking place as a result of this treatment. Further studies are in progress to determine the various factors that can affect T_1 during the cell cycle.

This study opens up a new line of in-

vestigation for the examination of the physical properties of water and its interactions with macromolecules during the complex biochemical and physiological processes of cell growth and division.

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Vasoactive Intestinal Polypeptide: Abundant Immunoreactivity in Neural Cell Lines and Normal Nervous Tissue

Abstract. *Vasoactive intestinal polypeptide immunoreactivity is present in high concentrations in clonal lines of neuronal and glial origin. The central nervous system and sympathetic ganglia are also rich in the peptide. The findings suggest that this peptide, hitherto thought limited to the gastrointestinal tract, is widely distributed in neural tissue and may have broad physiological significance.*

Originally isolated from porcine duodenum (*1*), the vasoactive intestinal polypeptide (VIP) is a 28-residue peptide that is structurally and biologically related to secretin and glucagon (*2*), and is found throughout the gastrointestinal tract of mammals and birds (*3*). The peptide may also be secreted by a variety of tumors (*4*), including some of neurogenic and neuroendocrine origin (*5*). The latter finding prompted us to search for VIP in cloned tumor cell lines of neural origin and in normal nervous tissue. We found high levels of immunoassayable peptide in clonal neuroblastoma and astrocytoma cell lines, of neuronal and glial origin, respectively. Vasoactive intestinal polypeptide, or a peptide that cross reacts with it, was also present in normal brain tissue, with the highest concentrations in cerebral cortex, and the lowest in cerebellum and brainstem.

Neuroblastoma cell lines, derived from the transplantable, C 1300 mouse neuroblastoma (*6*), comprised three clones: NE115, which is adrenergic; S20, which is cholinergic; and C46, which is neither adrenergic nor cholinergic (gifts of Dr. Marshall W. Nirenberg, National Institutes of Health, Bethesda, Maryland). The glial cell line was the C6 rat astrocytoma clone (*7*) (gift of Dr. Gordon Sato, University of California, San Diego). Cell monolayers were grown in Dulbecco's modified Eagle's medium, containing 10 percent fetal calf serum plus 200 μg of kanamycin per milliliter and 125 μg of spectinomycin per milliliter. Cultures were grown in Falcon flasks or tissue culture dishes at 37°C in an atmosphere of 10 percent CO_2 in air, at 100 percent humidity. Cells from exponentially growing cultures of each line were inoculated into a series of 100-mm tissue culture dishes. At specified intervals, one plate from each line was scraped off and the cells were counted (Coulter counter), suspended in 2 ml of buffer (0.05*M* KH_2PO_4, 0.001*M* ethylenediaminetetraacetate, adjusted to *p*H 7.3 with KOH), and sonicated before assay of the peptide.

Samples of normal neural tissue were taken from different parts of the brain, peripheral sympathetic chain, and vagus nerve. These samples were removed from dogs within 1 hour after exsanguination, and were extracted in dilute acetic acid or acid alcohol. Peptides were concentrated from these extracts by adsorption to alginic acid, followed by elution with 0.2*M* HCl and salting out (*1*), or by precipitation with ether.

Vasoactive intestinal polypeptide immunoreactivity was measured by a highly specific radioimmunoassay (*4*), which has been improved to detect 50 pg of the porcine peptide per milliliter. All samples were assayed in duplicate, and the assay was performed at least twice. In this assay, antibodies to VIP showed minimal ($< 1 : 1000$) or no cross-reaction with secretin (GIH Laboratory, Karolinska Institute, Stockholm), glucagon (Eli Lilly), cholecystokinin-pancreozymin (GIH Laboratory), bradykinin (synthetic, Sandoz), substance P (synthetic bovine, Beckman), or somatostatin (synthetic ovine, Beckman).

Cells from all three neuroblastoma lines were rich in VIP (Table 1), with a concentration ranging from 0.6 ng per million cells (or 2.2 ng per milligram of protein) to 0.9 ng per million cells (or 3.6 ng per milligram of protein). In each case, as the cell counts increased between the second and fifth days, total VIP levels also increased, although the

Table 1. Concentrations of VIP in neuroblastoma and astrocytoma cell cultures.

Days from inoculation	Neuroblastoma						Astrocytoma	
	Clone NE115		Clone S20		Clone C46		Clone C6	
	Cells ($\times 10^6$/plate)	VIP (ng/plate)	Cells ($\times 10^6$/plate)	VIP (ng/plate)	Cells ($\times 10^6$/plate)	VIP (ng/plate)	Cells ($\times 10^6$/plate)	VIP (ng/plate)
2	5.5	6.2	4.5	5.8	4.9	5.7	5.6	5.6
5	27.4	16.0	15.6	13.2	26.6	17.4	30.6	6.5