Physiol. Chem. Phys. & Med. NMR (2005) *37*: 141–158

What Befalls the Proteins and Water in a Living Cell When the Cell Dies?

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Abstract: The solvency of solutes of varying molecular size in the intracellular water of freshly-killed Ehrlich carcinoma cells fits the same theoretical curve that describes the solvency of similar solutes in a 36% solution of native bovine hemoglobin — a protein found only in red blood cells and making up 97.3% of the red cell's total intracellular proteins. The merging of the two sets of data confirms the prediction of the AI Hypothesis that key intracellular protein(s) in dying cells undergo(es) a transition from: (1) one in which the polypeptide NHCO groups assume a fully-extended conformation with relatively strong power of polarizing and orienting the bulk-phase water in multilayers; to (2) one in which most of the polypeptide NHCO groups are engaged in α-helical and other "introvert" conformations (see below for definition) with much weaker power in polarizing-orienting multilayers of bulk-phase water. This concordance of the two sets of data also shows that what we now call **native** hemoglobin — supposedly denoting hemoglobin found in its natural state in **living red blood cells** —, in fact, more closely resembles the water-polarizing, and -orienting intracellular proteins in **dead cells**. Although in the dead Ehrlich carcinoma cells as well as in the 36% solution of native hemoglobin, much of the protein's polypeptide NHCO groups are engaged in α-helical and other "introvert" conformation (Perutz 1969; Weissbluth 1974), both systems produce a weak but nonetheless pervasive and "long-range" water polarization and orientation. It is suggested that in both the dead Ehrlich carcinoma ascites cells and in the 36% native bovine hemoglobin solution, enough polypeptide NHCO groups assume the fully-extended conformation to produce the weak but far-reaching multilayer water polarization and orientation observed.

KEY WORDS AND TERMS: active living state, *ad infinitum* adsorption, association-induction hypothesis, AI Hypothesis, ATP as EWC, auto-cooperative transition, cardinal adsorbent, cell physiology, dead state, Ehrlich ascites cells, electron-withdrawing cardinal adsorbent, EWC, fully-extended protein, gelatin, hemoglobin, the living state, long-range dynamic structure of water, q-value of NaCl, native hemoglobin, denatured hemoglobin, non-evaporating water, non-freezing water, polarized-oriented multilayer theory, PM theory, poly(ethylene glycol), polypeptide, protein hydration, protoplasm, red blood cell, resting living state, ρ-value, Size Rule, water,

IN THE CONVENTIONAL membrane-pump theory, a living cell represents a membrane-enclosed solution of free solutes and the so-called *native* proteins in ordinary liquid water. Incessant activities of a battery of submicroscopic pumps postulated to reside in the cell membrane determine largely the cell's chemical makeup. On cell death, the pumps stop pumping. The asymmetric molecular and ionic distribution dissipates and the cell water and its dissolved substances mix with, and become an indistinguishable part of the cell's dead aqueous environment. As such, the theory does not anticipate any profound changes in the cell proteins nor in the bulk-phase cell water.

However, this membrane-pump theory has long ceased to be a valid theory. The details of its exhaustive disproof, reviewed again and again (see Ling 1997, and Chapter 12 of Ling 2001) are, unbelievable as it might seem, virtually unknown to the great majority of those who would like to know or should know.

However, it is not all darkness. There is also a brighter side. That is, at this very moment, there already exists (though also hidden) a theory of cell physiology, which has already reached maturity and successfully stood over 40 years of world-wide experimental testing.

This verified, new unifying theory bears the title, the **Association-induction hypothesis** or **AI Hypothesis** for short. Its continuing growth and leap-frog confirmation have been documented in four full-length monographs, whose publication span four decades (Ling 1962, 1984, 1992, 2001.)

In the following, we shall begin by reviewing briefly the relevant parts of the association-induction hypothesis that describe what and why this theory predicts that there is a profound change in the conformation of the key cell proteins and in the physical state of the bulk phase water when a cell dies.

Theory

Historically, the membrane (pump) theory has been by far the dominent theory of the living cell. However, Martin Fischer (1908, 1909); Benjamin Moore and Herbert E. Roaf (1908); Moore, Roaf and Webster (1912); W.W. Lepeschkin (1928); Ross A. Gortner (1930); E. Ernst (1963, see also Ernst and Scheffer, 1928); Dimitri N. Nasonov (1930, 1962) and A.S.Troshin (1966) all have their own ideas or theories on one subject or another. In addition to those specific ideas, they all shared a skepticism about the validity of the membrane-pump theory and a belief in the importance of protoplasm, which once Thomas Huxley eloquently called the physical basis of life (Huxley 1869.) One reason for the dominance of the membrane (pump) theory was that it was or at least appeared to be a coherent general or unifying theory. That is, until it was proven altogether wrong and a new unifying theory, the association-induction hypothesis, emerged and gradually affirmed.

In introducing and further developing the concept of a "living substance" or protoplasm, Ling's association-induction hypothesis is itself a continuation of the work of these deserving but alas, almost forgotten investigators, whose names are mentioned above and others who are unintentionally left out. Coming late on the scene — as it was the case with the association-induction hypothesis —, also has provided an advantage denied all

our predecessors: the great progress that had been made in fundamental physics and chemistry as well as biological sciences. It is obvious that without its help, the association-induction hypothesis would not have come into existence.

In the association-induction hypothesis, the early concept of protoplasm being the physical basis of life is fully retained. However, major changes have also been made and new concepts incorporated, so that the glaring weaknesses and mistakes in the old definition of protoplasm that have played a part in its misconceived total rejection, have been replaced to become a part of the self-consistent and coherent theory called the AI Hypothesis.

Thus, in the association-induction hypothesis, being alive signifies the existence of a closely-associated system of proteins-water-ions (or protoplasm), cooperatively-linked in a high (negative) energy and low-entropy state called the **living state**. An implication of this new definition is that just as a cell can live or die, so can the protoplasm making up different parts of the living cell. Cytoplasm, cell (or plasma) membrane, nucleus, nucleolus, Golgi apparatus, mitochondrial cristie, ribosome are the names given in the past to some of the protoplasmic components of the living cell.

As such, protoplasm is not just a "substance" defined by an unvarying chemical composition, no more so than a rainbow is merely a collection of different colored bands. Rather, protoplasm is a unique substance containing as its major components, water, small molecules and ions like K^+ (and/or) Na^+ and ATP but above all, proteins, a class of macromolecules found only in, and made by living cells. When this unique assembly of proteins, water, K^+, ATP and other helpers exist in a (reversible) high-(negative)-energy, low-entropy state we call it the (resting) **living state** (Ling 1992, pp. 31–38; 2001, pp. 148–156.)

The maintenance of the protoplasm in the living state depends on the controlling influence of a consortium of small but critically important molecules or ions called **cardinal adsorbents** (Ling 2001, p. 166.) The cardinal adsorbent *par excellence* is, without question, the end product of energy metabolism, adenosine-triphosphate, or **ATP** mentioned earlier. In action, ATP is aided by what are collectively called **ATP helpers**. They include **congruous anions** (Ling 2001, p. 153) and an as yet-unidentified protein called **protein X** (see Figure 1) (Ling 2001, pp. 152–154.) Inconclusive preliminary search suggests that protein X might be or include *actin* (see Ling 2001, p. 154.) Together, ATP and its helpers keep the protoplasmic proteins in what I shall refer to as an open or **extrovert** conformation (illustrated in the right-hand-side picture in Figure 1) (see also Ling 1992, pp. 107; 2001, p. 77.)

To maintain the living state, ATP plays a *key* controlling role. For it is only when ATP is adsorbed on specific key sites on the protoplasmic protein called **cardinal sites** is the protoplasm alive. Only when ATP is abundant and suitably adsorbed are the negatively charged β-, and γ-carboxyl groups — carried respectively on aspartic and glutamic acid residues of the involved cell protein(s) — ready to preferentially adsorb K^+ ion over its alternatives: namely, Na^+ ion and fixed cations carried on lysine and arginine side chains (see right-side schematic illustration in Figure 1.) This explains why K^+ exists in much higher concentration in cells than in the surrounding medium and the bulk-phase water exists as strongly polarized and oriented multiple layers. And, why metabolic poisons like cyanide and iodoacetate kill cells by blocking the resynthesis of, and hence depleting ATP. Then K^+ is lost from the cell.

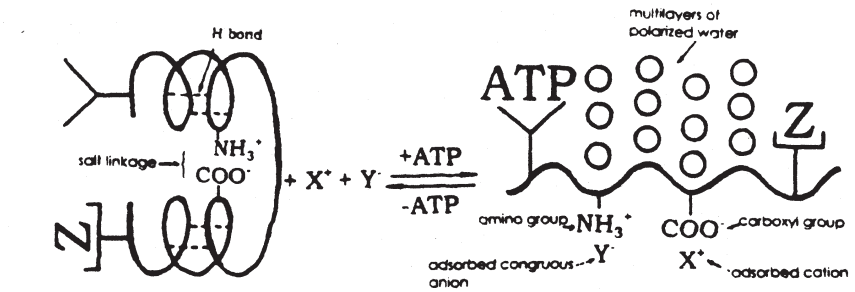

FIGURE 1. Diagrammatic illustration of reversible cooperative transition of a microscopic portion of living protoplasm. The transition is from an extrovert high (negative) energy — low entropy *resting living state* (right-hand side figure) to an introvert low (negative)energy — high entropy *active* or *dead state* (left-side figure.) This transition is under the control of the premier *cardinal adsorbent,* ATP — with the aid of *congruous anions* (Y^-) and *protein X*. In the extrovert conformation of the resting living state, the β- and γ-carboxyl groups are free to adsorb free ions X^+ (e.g., K^+) and the backbone NHCO groups are free to polarize and orient multi-layers of water molceules. In the introvert active or dead state, the β-, and γ-carboxyl groups are locked in salt-linkages with fixed cationic groups and the backbone NHCO groups form α-helical or other intra- or intermacromolecular H-bonds. (from Ling 2001)

In addition, Figure 1 also shows that in the *extrovert* conformation, a significant portion of the backbone carbonyl and imino groups of the protoplasmic proteins exist in the **fully-extended conformation (**Ling 1992, p. 121; 2001, p. 75.) The NH and CO groups of the fully-extended protein backbone polarize and orient multiple layers of water molecules near and far.

Assuming the dynamic structure (Ling 2001, p. 77) of **polarized-oriented multilayers**, the bulk-phase cell water has normal or reduced solubility for molecules and ions — collectively referred to as **solutes —** according to what is known as the **size rule** (Ling 1970, pp. 147–148; 1987; 1992, p. 77; 2001, p. 92.) That is, the larger the solute molecule, the smaller its *equilibrium distribution coefficient* or q-value in the cell or model water. Since hydrated Na^+ ion is large, its concentration in cell water is low. Nor can Na^+ ion successfully compete against K^+ ion for adsorption on the β-, and γ-carboxyl groups. Accordingly, Na^+ ion is found at low concentration in most normal living cells as observed. In the AI hypothesis, neither the selective accumulation of K^+ nor the partial exclusion of sodium ion from the normal resting cell requires a *continuous* expenditure of energy. Rather, they represent different aspects of a *metastable* equilibrium state. Nonetheless, the maintenance of this *metastable* equilibrium state requires ATP. However, it only depends on the presence of ATP *per se* and its adsorption as such on key *cardinal sites*. It does not require its continuing splitting and delivering a package of energy stored in the so-called high energy. Long ago, Morales and Podolsky (1956) have shown that no such high energy phosphate bond exists (see Ling 2001, Table 3 on p. 112.)

According to the AI hypothesis, when the cell dies, the protoplasmic **system** of proteins-water-ions undergoes a **cooperative transition** (Ling 1962, pp. 162–163; 1964, 1980, 2001, p. 295; Karreman 1980) from the high- (negative)- energy-low entropy **resting living state** (Ling 1962, title of book; Ling 1992, pp. 32–33; 2001, Figure 3.2 on p. 33) to a low-(negative)-energy, high-entropy **dead state**. The dead state is a further

dissipative step down beyond that of the **active living state** (see Figure 3.2 on p. 33 in Ling 1992.)

As illustrated on the left-side schematic diagram of Figure 1, the protein involved assumes in the dead state what is called an **introvert conformation**. In this conformation, the negatively-charged β-, and γ-carboxyl groups are no longer free but are engaged in *salt-linkages* with an equivalent number of fixed e-amino groups and guanidyl groups from lysine and arginine side chains respectively. In concert with the anchorage of the charged side chains brought about by salt-linkage formation, the backbone carbonyl and imino groups also become immobilized by engaging in intra- and/or inter-macromolecular H-bonds leading to the formation of the α-helical or β-pleated sheet conformation respectively. The transition from the *extrovert* to the *introvert* conformation in the dying cell liberates K^+ ions previously adsorbed singly on the β-, and γ-carboxyl groups; it also liberates water molecules adsorbed in polarized-oriented multilayers on the backbone CO and NH sites of the same or other intracellular proteins.

The progress made so far in establishing the **polarized oriented multilayer theory** (PM theory) **of cell water (**Ling 1965, 1970, 1972, 2003**)** — a subsidiary of the AI hypothesis — has involved the study of *inanimate models* side-by-side with the study of living cells. Chosen on the basis of fundamental principles of the PM theory, these models are divided into the two categories already alluded to above: **extrovert models** and **introvert models**.

Extrovert models comprise certain types of what has unfortunately been known as **denatured** proteins and oxygen-carrying linear polymers which exist in the full-extended conformation. Examples of extrovert models include urea, guanidine HCl and NaOH-denatured protein and nitrogen-carrying linear polymers, including poly(ethylene oxide), poly (vinyl methyl ether), polyvinylpyrrolidone and polyvinyl alcohol. For the newly gained prominence of polyvinyl alcohol (gel) in its spectacular confirmation of the PM theory, see Discussion below. (Also see Ling 1992, p. 81 for a description of Ling's theory why gelatin differs from most other proteins and that of his latest definition of *colloid*, which is the namesake of gelatin.) In contrast and irony, what we traditionally call native proteins are, as a rule, *introvert* models.

With these basic concepts in place, we now examine what the AI Hypothesis predicts on the conformation of the intracellular proteins and on the state of water in living cells before and after cell death: *in its resting **living state**,* some major intracellular proteins exist in the fully-extended extrovert state and the bulk-phase water in a cell resembles water in the presence of a suitable concentration of **denatured** proteins like gelatin and NaOH-denatured proteins; in a **dead** cell, some intracellular proteins assume the introvert state and the bulk phase water resembles water in the presence of a suitable concentration of the so-called **native** proteins.

In the last 35 years or so, a great deal of time has been spent in establishing that a host of cell physiological properties and behaviors, traditionally attributed to membrane pumps, membrane pore sizes, etc., actually originate from the distinctive properties of cell water (Ling 1992.) And in support of the theory, the investigators have been able to demonstrate highly similar behaviors and properties of bulk-phase water in living cells and bulk-phase water in the presence of extrovert models, but not so at all or weakly so by water in the presence of equal or even higher concentrations of introvert models of native proteins. (The interested reader should consult Chapter 5 of Ling 1992, especially

Table 5.5 for a complete summary of the extensive evidence referred to and the names of the investigators who created them.)

To put to a test the predicted similarity in the physico-chemical attributes of water in dead cells and water in the presence of (introvert) protein in the so-called native state, we select for testing the *solvency of the bulk-phase water for solutes of different molecular sizes*. This basic property of bulk-phase water was chosen on account of its simplicity and incisiveness in telling us *quantitatively* what is normal liquid water and what is not.

Now, hemoglobin makes up 97.3% of the total intracellular proteins of a mature mammalian red blood cell. Ideally, the comparison should be made, for example, between killed red blood cells and a solution of native hemoglobin at the concentration it is found in the red blood cell, i.e., 36%. However, there was a practical reason against it. At the time of our current study, we had not studied solute distribution in normal red blood cells. But we had completed the study of the solute distribution in three other kinds of living cells, frog muscle, frog ovarian eggs and Ehrilich carcinoma cells — though to this date, only the results on the frog muscle have been published (Ling *et al.* 1993.)

So, as the next best, we compared the solvency properties of a solution of native bovine hemoglobin — the study of which had also been more or less completed and eventually published (Ling and Hu 1988, 2004) —, with the solvency properties of killed Ehrlich carcinoma cancer cells.

Materials and Methods

(1) Cell preparation, incubation and extraction

Ehrlich carcinoma cells in the ascites form were carried in ICR mice and harvested 14 days after intraperitoneal inoculation. The ascites fluid collected was mixed with an equal volume of what will be referred to as the incubationn solution, i.e., a modified Kreb's Ringer solution (composition to be described below) and the suspension centrifuged at 500 rpm on an International Centrifuge for 5 minutes to separate the cancer cells from red blood cells, which remained suspended. For very bloody ascites fluids, this step might be repeated one or even more times. The weights of the ascites cells collected at the bottom of the pre-weighed centrifuge tubes were determined. An equal weight of incubation solution was added to produce a 50% cell suspension. A 2-ml aliquot of this 50% suspension was then diluted with 20 ml of an incubation solution in a 500 ml Erlenmeyer flask. To keep the pH near neutral, this flask was equilibrated with a mixture of 3% CO_2 and 97% O_2, or a mixture of 3% CO_2 and 97% N_2. The flasks were then shaken at 75 excursions per minute in a constant temperature bath maintained at 25°C. The incubation lasted 20 hours. The permeability barrier of the killed ascites cells are much weakened; this duration of incubation is more than long enough to insure distribution equilibrium even for very large solute molecules determined from prior study of the time of equilibration of the same solutes in intact Ehrlich cancer cells.

The incubation solution used was a modified Kreb's Ringer solution containing the following: NaCl (135 mM); KCl (5 mM); NaH_2PO_4 (1.16 mM); Na_2HPO_4 (1.8 mM); $NaHCO_3$ (3.6 mM); D-glucose (5.6 mM); $MgCl_2$ (0.9 mM); $CaCl_2$ (1.30 mM.) In addition, to the solution might also be added Penicillin G (0.01%), Streptomycin sulfate (0.01%.)

We killed the cancer cells by including in the incubation solution 1 mM NaCN and 5

mM iodoacetamide (which is a faster acting poison than sodium iodoacetate) replacing an equivalent amount of NaCl. To test the time required to kill the cells, we used a vital dye method (Erythrocin B, 0.28% in 0.154 M sodium chloride) and counted each time a total of 600 to 800 live and dead cells at 0, 1 and 2 hours of incubation. The results showed that at 0 hour incubation the total percentage of live cells was 91.7%. The surviving cells dropped precipitously to 3.05% after 1 hour of incubation, and to 0.84% after 2 hours of incubation respectively. These data reassured us that very early on during the 20 hour-long incubation, all or virtually all cells were dead.

In regular runs, the incubation solution contained probe solutes of different concentrations (replacing osmotic equivalents of NaCl in the incubation solution) in addition to 1 mM NaCN and 5 mM iodoacetamide. At the end of incubation, the cell suspension in the incubation solution was introduced into one or two 10-ml plastic syringe barrels. The tip of the syringe barrel was inserted snugly into the open end of a 500-microliter polypropylene micro-centrifuge tube. The assemblies were centrifuged at 2000 rpm for 10 minutes in an International Centrifuge. Aliquots of the supernatents were collected (for later analysis of its solute concentration) before the syringe barrels were separated from the microcentrifuge tubes. Now loaded with dead ascites cells, the microcentrifuge tubes were capped and centrifuged at 15,000 rpm for 10 minutes in a Sorval RC-2 centrifuge to get rid of most of the entrapped incubation solution, which was then aspirated off and discarded. The cells at the bottom of the microcentrifuge tubes were divided into two portions.

One portion of the separated cells was introduced into pre-weighed aluminum pans. The dry weight and water content of the cells were obtained by weighing the loaded pan before and after drying in an oven at 100°C for 24 hours. The other portion of cells was blown into preweighed 15 ml Nalgene centrifuge tubes and the loaded tubes weighed again to obtain the wet cell weight. 3 ml of a 5.1% trichloroacetic acid was added to each tube and mixed with the aid of a Vortex shaker. The tubes were kept overnight in a cold room for solute extraction before centrifugation at 5000–10000 rpm for 5 to 10 minutes to spin down the precipitated cell debris. The clear supernatant solutions were diluted before assaying for the concentration of the probe solutes in the cell water.

(2) Assay of non-radioactive and radioactive solutes.

For solute extraction from cells, we used either 5% trichloroacetic acid (TCA) already mentioned above, or boiling water extraction followed by deproteination with the aid of $Ba(OH)_2$ and $ZnSO_4$ according to the method of Somogyi (1930.) L-lyxose was determined by the method of Roe and Rice (1948.) D-mannitol, D-sorbitol and perseitol were determined by the method of Burton (1957.) Sucrose, raffinose, and melezitose were analyzed for their reducing-sugar content by the method of Roe (1934) after hydrolysis by heating for 10 minutes at 80^{o} C in an extract containing 21% HCl. For assay of radioactively labeled solutes, TCA extracts or supernatant containing similar TCA concentration were mixed with Bray's scintillation fluid (Bray 1960) and assayed on a beta-scintillation counter according to the method described by Ling and Hu (1988.)

(3) Determination of the extra-cellular space

It is not easy to determine accurately the extra-cellular space in dead ascites cells because the probe solutes often used to estimate the extracellular space of a population of normal cells do not stay outside the cells but penetrate into the cell interior to varying degrees.

Thus a compromise was chosen. The compromise involves the following assumption. The extracellular space in a centrifuged collection of dead cancer cells is the same as that for a similar population of live cancer cells spun down for the same length of time at the same gravitational force and in the same kind of centrifuge tubes.

To obtain the extracellular space of a population of live Ehrlich carcinoma ascites cells we first cleared the cell suspension of red blood cells by the centrifugation procedure described above. The ascites cancer cells spun down were then suspended in the modified Kreb's Ringer solution containing approximately 0.02 microcuries of ^{3}H-inulin to give approximately 20,000 counts per minute when assayed in the beta-counter. After mixing, the cells were introduced into the same type of syringe barrel-microcentrifuge assembly described above. The assembly was then spun down at 2000 rpm for 4 minutes and aliquots of the supernatant collected and its radioactivity assayed. Cells in the microcentrifuge tubes were spun down at 15,000 rpm for 10 minutes in the same Sorval RC-2 centrifuge used for other studies described above. Resuspended into 2 ml of Kreb's solution, the cells were then quickly spun down and aliquots of the supernatant solution weighed and counted. From the counts of the two supernatent solutions and the weight of the cells resuspended, we obtained for eight samples the following weight percentages of fluid in the extracellular space: 4.0; 5.6; 5.0; 6.7; 6.9; 4.6; 5.0; and 5.1, averaging 5.4% ± 1.0 % (mean ± SD).

(4) Calculations of solute concentration in cells and its q- or ρ-value

From the determination of the water content, the non-radioactive or radioactive solute concentration in the total water content of the cell pallet ($[S]_{cp}$) and in the water of the supernatant solutions ($[S]_s$), the solute concentration in the cell water all in units of micromoles per ml or weight percentage and represented as $[S]_{cw}$ is calculated according to the following equation:

$$[S]_{cw} = \{[S]_{cp} - 0.063\ [S]_s\}/\ \{1 - 0.063\}. \quad (1)$$

Next, we explain the meaning of the figure, 0.063. As mentioned above, we obtained from radioactive inulin assay, an average of extracellular space of 5.4%, which we rounded off to 5% in grams of water per gram of *whole cells*. To convert this figure to that of percentage volume of extracellular space per gram of *cell water*, the 5% figure is divided by the average water content, equal to 80% and we obtained the figure, 0.063 cited above.

The final goal of our calculations was to obtain either the *true equilibrium distribution coefficient* or **q-value** or failing that, the *apparent equilibrium coefficient* or **ρ-value**. Under the most favorable conditions, the q-value and ρ-value are equal. The q-value, of course, equals $[S]_{cw}$ divided by $[S]_s$. Note that $[S]_{cw}$ refers exclusively to solute in the cell water and it does not include solute that might be adsorbed on the cell proteins for example. The usual criterion to determine that a solute inside a cell is exclusively or nearly exclusively in the cell water is its rectilinear distribution. Any significant amount of adsorption would cause the distribution curve over a wide range of concentration to deviate from that of a straight line. Therefore, unless we have demonstrated such a straight line in the data from a spread of experimental points in a plot of $[S]_{cw}$ against $[S]_s$, the ratio obtained would be designated as a ρ-value.

(5) Sources of the values of molar volumes or molecular volumes

The molar volume or molecular volume is equal to the molecular weight of a solute divided by the density of that solute in its liquid state near its boiling point. None of the solutes we used for this study exists in the liquid state at ambient temperature and no such density data are available to make a precise determination of the molecular volume possible. As reported in a preceding paper (Ling *et al*. 1993), when liquid density is available even though it is not near the solute's boiling point, we used it anyway to calculate an approximately correct value. When liquid density (at any temperature) is not available, we used the solid density, or failing that, we computed molecular volumes as the sum of the "volume equivalents" of the elements as given by Kopp: hydrogen, 5.5; carbon 11.0; oxygen (-O-), 7.8, each multiplied by the number of atoms of that respective element in the solute molecule (see Glasstone 1946, p. 525.)

(6) Chemicals

We obtained the following chemicals from Sigma Chemical Co., St. Louis, MO : L-lyxose, Catalog No. L-7126, Lot 26F-0479; D-sorbitol, Catalog No. S-1876, Lot 34F-0016; D-mannitol, Catalog No. M-4125, Lot 81F-0517; D-melezitose, Catalog No. M 5375, Lot 35F-0328; iodoacetamide, Catalog No. I 6125, Lot 36F-5626, inulin (from dahlia tuber) Catalog No. I-3954, Lot 66c-7130, poly(ethylene glycol) (M.W. 3300) Catalog No., P 3640, Lot 16F-0477. The following two chemicals were from Aldrich Chemical Co., Milwaukee, WI., perseitol, Catalog No. 13661-1; D- raffinose Catalog No. 20667-9, Lot. 00302 BM. From Fisher Scientific Co., we obtained sucrose, Lot 851322. Labeled ^{3}H-inulin, Batch 1496-222 and labeled poly(ethylene glycol) [1,2-^{14}C] (M.W. 4000), Batch 1291-178 were both from New England Nuclear, Boston, MA.

Results

In Figure 2, the q-value and/or ρ-values of solutes in the water of dead Ehrlich ascites cells are represented as open circles and plotted against their various molecular volumes. Since the solutes with molar volume smaller than 1000 cc are all more or less constant in their equilibrium distribution coefficient at around 1.0 and to save space, only a duplicate set of data were obtained from all of these smaller solutes (see Table I.) However, for solutes with molar volume larger than 1000 cc, the equilibrium distribution coefficients are altogether different; they fall on a steep declining curve and small errors which would have no significant impact on the lower end of the spectrum, would have marked adverse effect here. To avoid that, all the data collected on the equilibrium distribution coefficients of probe molecules larger than 1000 cc are *true equilibrium distribution coefficients* or q-values; they were obtained from more experimental points covering a wide range of the probe molecule concentrations as indicated in Table II and plotted in Figure 3.

In Figure 2, we also replotted the q-values — represented as solid triangles — of similar and different solutes in a solution containing 36% (w/v) of native bovine hemoglobin previously reported by Ling and Hu (Ling and Hu 1988, 2004.)

The solid line that runs reasonably close to **both sets** of data points is the same theoretical curve computed in 1992 according to Ling's theoretical equation with variable

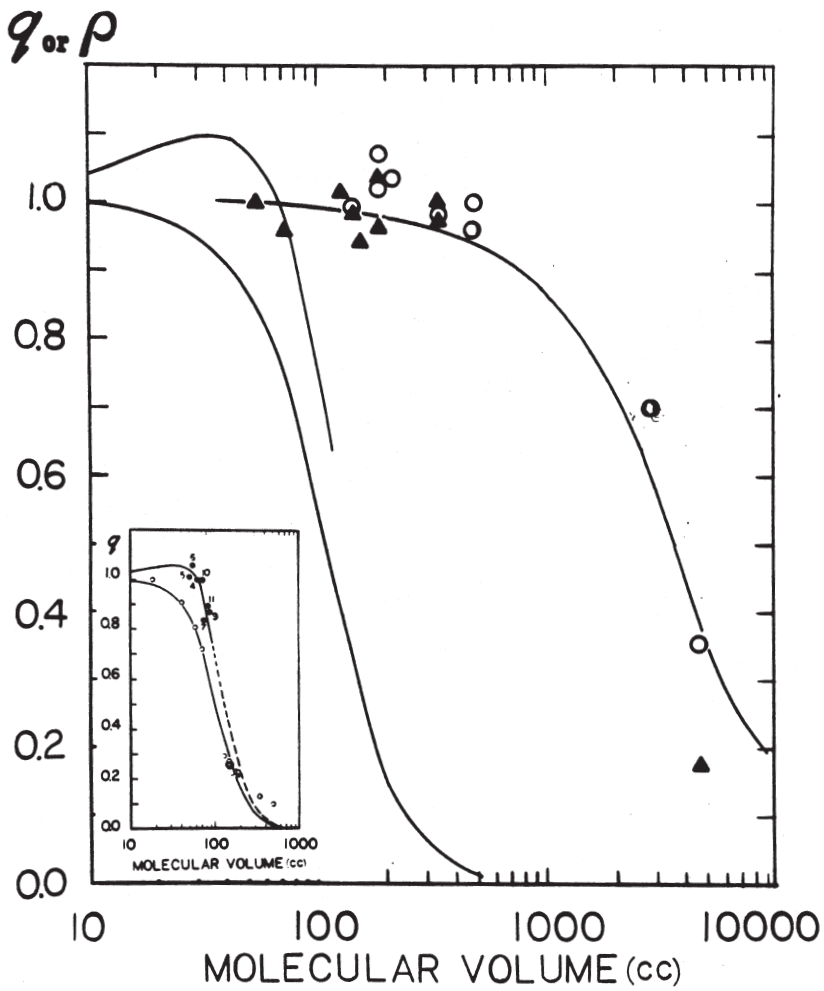

FIGURE 2. The q-values or ρ-values of neutral solutes of different molecular volumes in a solution of 36% native hemoglobin (solid triangles) and in the water in dead Ehrlich ascites cells (open circles.) The q- or ρ-values are plotted against the respective molecular volumes of the solutes. The native protein data as well as the theoretical curve based on Equation A1 in Endnote 1 drawn originally to fit the native hemoglobin data were from previously published data of Ling and Hu (1988 and 2004) and from Ling *et al.* (theoretical curve 1993) respectively. The inset showing q *vs* v plot of living frog muscles and the outlines of the theoretical curves shown in the inset are also drawn on the main graph to accentuate the profound difference between the solute-distribution profiles of live and dead cells. (It should be pointed out that there is an insignificant small difference in the $^{0}u_{vp}$ used to calculate the right-hand side curve (3.8 cal/mole) and $^{0}u_{vp}$ used in a prior publication of Ling and Hu (2004) from a later more up-to-date data on the q-value of PEG-4000 in a 36% hemoglobin solution (4.25cal/mole). This 3.3% difference is trivial and to be ignored.)

TABLE I. The q-values or ρ-values of solutes of different molecular volumes of water in dead Ehrlich ascites cells.

Solute	Water content (%)	Mol. Vol. (cc.)	ρ or *q**
L-Lyxose	88.1	149	0.99 (0.99; 0.98)
D-Sorbitol	86.4	189.8	1.02 (1.04;1.00)
D-Mannitol	86.3	189.8	1.07 (1.06; 1.08)
Perseitol	87.0	219.6	1.03 (1.05; 1.00)
Sucrose	86.0	338.8	0.98 (1/02; 0.93)
Raffinose	85.2	443.8	0.96 (0.96; 0.96)
Melezitose	84.7	498.8	1.00 (0.96; 1.01)
Inulin	83.2 ± 0.49	2592	0.52 ± 0.039 (4)*

The q-value of inulin from our earlier studies is lower than from the larger set of data given in Table 2 (0.698), which alone is presented in Figure 2.

TABLE II. The true equilbrium distribution coefficient or q-values or apparent equilibrium distribution coefficient or ρ-values of inulin and PEG-4000 in freshly killed Ehrlich carcinoma cells in the ascites form.

Solute	Mol. Vol. (cc.)	H_2O (%)	$[S]_{ex}$ (%)	$[S]_{in}$ (%)	q
		85.2	0.092	0.060	
		82.6	0.091	0.063	
		84.3	0.340	0.213	
		85.3	0.340	0.211	
		82.5	0.590	0.411	
		81.8	0.610	0.400	
		81.9	0.840	0.602	
inulin	2592	82.2	0.850	0.583	0.69
		83.0	0.088	0.058	(+ 0.998)
		81.2	0.090	0.061	
		80.1	0.350	0.23	
		19.9	0.340	0.22	
		78.3	0.610	0.43	
		79.1	0.600	0.43	
		82.5	0.860	0.59	
		81.5	0.870	0.58	
		80.7	0.95	0.31	
		80.0	1.10	0.37	
PEG-4000	4717	75.8	2.71	1.11	0.35
		77.4	4.87	2.24	(+ 0.946)
		78.0	5.48	2.02	
		76.7	5.67	1.86	

The respective q-values were determined from the slopes of the best fitting curves. The number in parentheses following are the linear correlation coefficients between the intra- and extra-cellular concentrations. The dahlia tube inulin we used had a wide spread of molecular weights between 2000 and 5000. We obtained these figures from Sigma Company's 2004–2005 catalog on the item dahlia-tuber inulinF1TCA. To obtain the molecular volume we divided the average of the two limits (3500) by the solid density of inulin of 1.35 from Hodgman *et al*, 1961, p. 1051) to obtain the figure of 2592 cc.

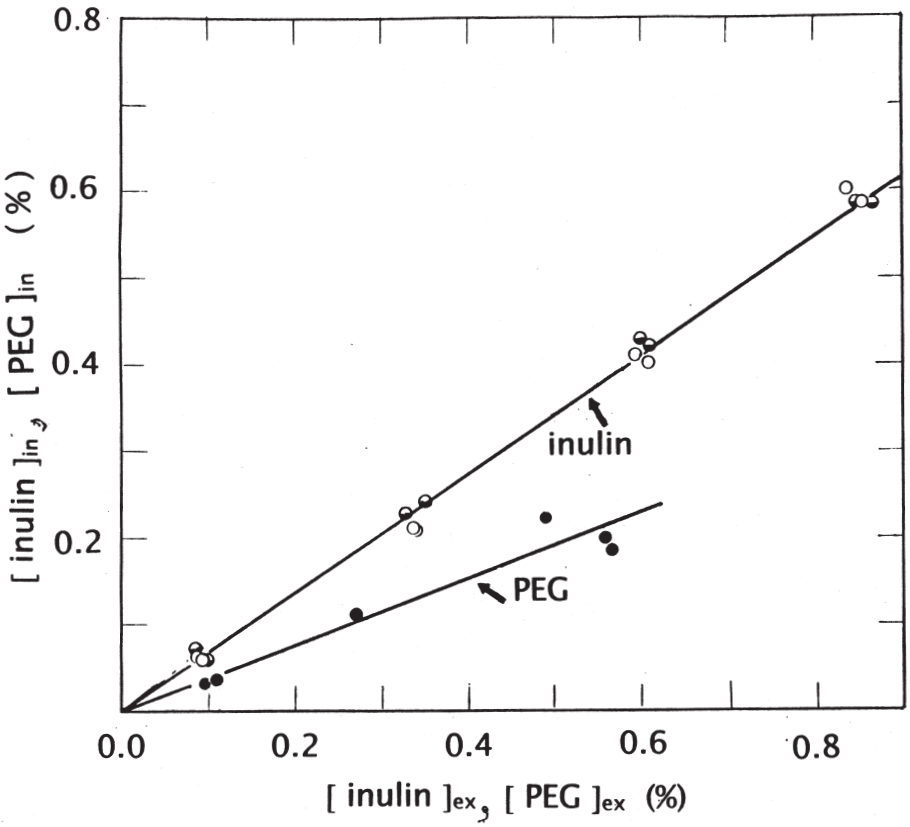

FIGURE 3. The equilibrium distribution of inulin and PEG-4000 in freshly killed Ehrlich carcinoma cells (25°C.) To kill the cells, freshly isolated cells were exposed to a Kreb's Ringer solution containing 1 mM NaCN and 5 mM iodoacetamide. As indicated in the graph, both the inulin concentration and that of PEG-4000 were in units of weight percentages. Numerical values are the same as those shown in Table II.

parameters chosen to fit best the solute distribution data points in water containing 36% native bovine hemoglobin. Ling's theoretical equation is given as Equation A1 in Endnote 1.

Conclusion and Discussion

The theoretical curve originally computed according to Equation A1 in Endnote that fits the set of the data from native hemoglobin and represented as solid triangles also fits the second set of data from dead Ehrlich carcinoma cells represented as empty circles. This agreeement leads to two conclusions: (1) Water in dead Ehrlich ascites cells resembles water in the presence of suitable concentration of native protein, as predicted by the AI hypothesis. (2) Within the boundary of the experimental error, both sets of data behave in a way according to the PM theory of solute distribution expressed in Equation A1 in Endnote embodying the "size rule".

To give context to the observed similarity of water in the presence of native hemoglobin and in killed Ehrlich ascites cells we make three comparisons and one comment:

(1) Comparing water in dead Ehrlich carcinoma cells with water in a 36% solution of native hemoglobin

Up until 2003, no theory could explain the three sets of amazing observations on how special solid surfaces could alter profoundly the properties of truly deep layers of water molecules. They are GiguPre and Harvey's unexpected demonstration of 10 micra thick layer of nonfreezing water held between polished AgCl prisms at the lowest temperture they could administer, −176°C (Giguère and Harvey 1956); Hori's demonstration of 300 Å thick layer of water film held between plates of polished glass, would not evaporate at 300°C (Hori 1956) and Tseng and Pollack's demonstration of exclusion of coated microsphers from water 100 micra or even further away from a solid polyvinyl alcohol gel (Tseng and Pollack 2003.) Each of these studies revealed respectively that water under the influence of suitable solid surfaces would not freeze, evaporate or exclude microsphers at distance far away from the solid surface. And then Ling offered his new theory of long range water polarization-orientation, which predicts each of these three unusual behaviors: non-freezing, non-evaporating at 300°C and exclusion from water 0.25 mm. away from the polarizing surface (Ling 2003.) For the theory predicts *ad infi*nitum polarization-orientation of water under *ideal* conditions by an *idealized* NP surface with a water-to-water interaction energy stronger than that in ice and also strong enough to resist evaporation at 300°C.

However, of direct importance here is the fact that the suitable NP surface can make the water molecules assume an altered configuration and once this alteration has happened, the alteration would spread on by itself. In other words, except at the first and second layer of water molecules right next to the polarizing-orienting ideal or near-ideal NP surface, the high (negative) water-to-water interaction energy is uniform over a large volume of water involved.

As a matter of fact, this uniformity of water-to-water interaction energy was one of the fundamental assumptions in Ling's theory of size-dependent solute exclusion following the size rule (Ling 1993.) And it was discussed by Ling and Hu in an earlier paper on the exclusion of PEG-4000 from the 36% hemoglobin solution: that is, the q-value of 0.189 does not signify that 81% of the water in the 36% hemoglobin has zero solvency for PEG-4000 and the remaining 18.9% has normal 100% solvency. Rather, the entire amount of water in the 36% hemoglobin solution has uniformly reduced q-value of 0.81 for PEG-4000. The fact that the solute distribution data in dead Ehrlich cancer cells can be described by the same theoretical curve describing that of the 36% hemoglobin solution would suggest that the bulk-phase water has also a uniformly reduced solvency. In other words, all the water in the killed cancer cells are not normal liquid water plus a small amount of altered hydration water but polarized-oriented by a volume component of excess water-to-water intraction energy or ∂u_{vp} of some 4 cal/mole excess water-to-water interaction energy throughout (see Endnote 1 for a more detailed explanation of ∂u_{vp}.)

(2) Comparing water in live and dead cells

To underscore the profound difference between water in living cells and water in dead cells, we have included in an inset of Figure 2, solute distribution data in living frog

muscles published earlier and the two sets of theoretical curves fitting what one may call ordinary solutes (lower curve) and solutes most of which are known cryoprotectants (lower curve.) According to the polarized-oriented multilayer theory or PM theory, which is a subsidiary of the AI Hypothesis, cryoprotectant molecules have surface attributes making them more closely interacting with, and thus making stronger the normal cell water's dynamic multilayer polarized-oriented dynamic structure. The outlines of these two sets of theoretical curves are also reproduced on the main graph of Figure 2. To underscore the profound change of the bulk-phase cell water upon death, we make the following quantitative comparison: While a solute with a molecular volume of 300 cc already has a q-value below 0.1 in the intracellular water of a living cell (frog muscle) (Ling *et al*. 1993); a solute with a molecular volume of more than three-times larger still has a q- (or ρ-value) in the realm of 0.9 in the intracellular water of a dead cell.

The cause for this change in the solute-distribution pattern seen on cell death is a pervasive and drastic loosening of the dynamic water structure. Thus, the theoretical curves which fit the living frog muscle data with a $\mathcal{U}_{vp}$ (the volume component of the excess water-to-water interaction energy) of 126 cal per mole. In contrast, the theoretical curve computed best to fit the native hemoglobin data (now shown to also fit the dead cell data), was computed on the basis of a corresponding $\mathcal{U}_{VP}$ of only 3.8 cal per mole, thus detecting a 32-fold decrease in the excess water-to-water interaction energy due to multilayer polarization and orientation.

Of course, it would be even more cogent to compare water in live Ehrlich ascites cells with water in dead Ehrlich ascites cells. As mentioned earlier, a study of solute distribution in living Ehrlich ascites cells have also been completed. In anticipation of what we will publish in the future, we mention that by and large, the data on living Ehrlich ascites cells are not too different from that in normal living frog muscle cells already in print and reproduced here in Figure 2.

It is remarkable that physiological phenomena like solute distribution are so exquisitely sensitive to such minute differences in the volume component of the water-to-water interaction energy, $\mathcal{U}_{vp}$. A difference of 3.8 cal/mole of water, or 0.0038 Kcal./mole, is able to keep a molecule with a molecular volume of 4700 cc "off limit" from some 80% of the bulk-phase water. This sensitivity becomes even more striking if one recalls that the normal average water-to-water interaction described by the enthalpy of vaporization of liquid water is 9.7171 Kcal/mole (Rossini *et al* 1952), a figure more than 2500 times greater than the difference of 0.0038 Kcal/mole.

Perhaps this comparison offers some insight into just how extremely delicate and energy-efficient the living machines really are (Endnote 2.) After all, how else can a dung beetle fly almost as well as, or in some ways, even better than a gasoline-propelled airplane, when the dungbeetle's food is mostly grass, not ordinary grass, but grass from which the wildebeest and his stable of intestinal fauna and flora have extracted the most available nutrients?

(3) Comparing water in a solution of native protein with water in a solution of the same protein after its denaturation with NaOH.

In Figure 4 we reproduce a slightly modified version of a figure from Ling and Hu (1988.) In this figure, the size-dependent solute distribution profile of an 18% NaOH-denatured hemoglobin solution as well as the strikingly different profile of that of a 36% native

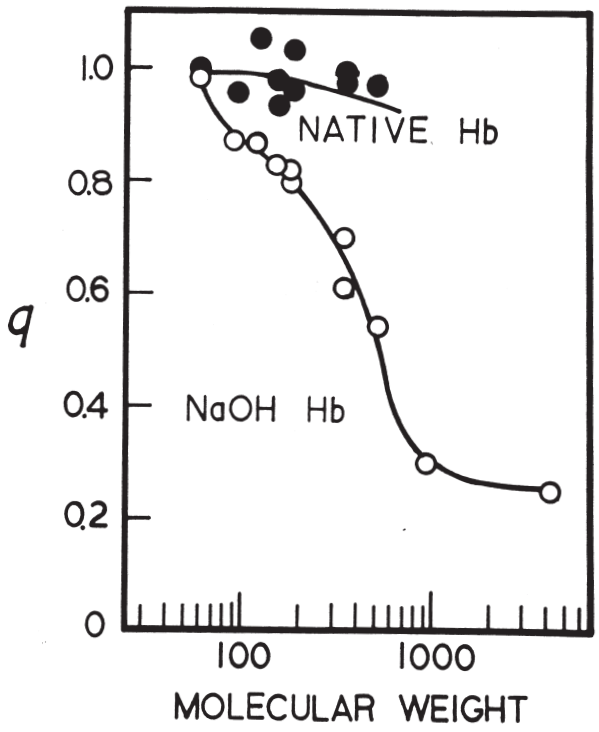

FIGURE 4. Plot of q-values of solutes of different molecular weights of solutes in the presence of NaOH-denatured hemoglobin (18%) (w/v) and an incomplete profile of q *vs* v from native hemoglobin. Data reproduced from an inset in an older publication to illustrate the different profiles of solute distribution in water under the influence of native protein and NaOH-denatured proteins. This graph is an older one made at the time when our q-value data shown in Figure 2 and 3 were still not available. (from Ling *et al.* 1993)

hemoglobin solution are given. We already know that the $\mathcal{U}_{VP}$ of the water in the 36% native hemoglobin solution to be 3.8 cal/mole. At 18% concentration, the excess water-to-water interaction energy in the bulk phase water of the NaOH-denatured hemoglobin solution is only one half the strength of the native hemoglobin solution. Yet its $\mathcal{U}_{VP}$ is 26.6 cal/mole and thus seven times higher than that in the *native* hemoglobin solution. The living frog muscle cell contains about the same concentration of proteins (20%) as the NaOH-denatured hemoglobin just mentioned, yet it has a $\mathcal{U}_{vp}$ nearly five times higher still than water in the presence of NaOH-denatured hemoglobin. Ling *et al* had discussed the various causes that could have created this difference; the most important is probably the much higher degree of order of proteins in protoplasm than in a denatured protein solution (Ling *et al.* 1993.)

(4) The weak but nonetheless long-range polarization of water by native proteins

The weak but multilayer polarization of cell water in the presence of 36% native bovine hemoglobin was a subject already discussed in the companion paper by Ling and Hu (2004.) We only need to mention the essence of the tentative conclusion they reached: although a

large portion of the polypeptide NHCO groups of the native hemoglobin is engaged in the α-helical conformation, there is reason to suspect that the percentage of the polypeptide chains engaged in α-helical and other introvert conformation in a 36% native hemoglobin solution may not be quite as large as those derived from crystallographic studies and that a significant part of the polypeptide chains may actually adopt the fully-extended conformation leading to the weak but non-the-less multilayer polarization-orientation of the bulk-phase water as observed.

We thank Dr. Raymond Damadian and his Fonar Corporation and its many friendly and helpful members for their continued support. We also thank Margaret Ochsenfeld and Dr. Zhen-dong Chen for their dedicated and skillful cooperation, and librarian Anthony Colella and Michael Guarino, director of Media and Internet Services for their patience and tireless assistance.

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Received November 11, 2005;
accepted December 23, 2005.

Endnotes

(1) Theory of q-value

The theory of solute distribution in cell water and water in model systems was a subsidiary of the PM theory of cell water, which in turn is a subsidiary of the AI hypothesis. Using standard statistical mechanical method, a quantitative expression of the theory of solute distribution in an equation form was first presented in 1993 (Ling 1993) and is given below as Equation A1

$$q = \exp\left\{\frac{1.23v\Delta E_s\left[1-(1-b)\dfrac{(kv)^n}{1+(kv)^n}\right]-(\Delta E_v+1.23\Delta e^*)v}{RT}\right\}, \qquad (A1)$$

Here the symbol v, represents the molecular volume of a solute under study; the symbol, ΔE_s represents the *specific surface (or solute) polarization energy per cm*^{2} when the solute is transferred from normal liquid water to the polarized cell water or model water. ΔE_v is the *specific volume (or solvent) polarization energy* per cm^3; it is equal to the difference in energy spent in excavating a hole 1 cm^3 in size in the polarized water and the energy recovered in filling up a hole of the same size in the surrounding normal liquid water. Δe^* is the *increment of the activation energy* for overcoming the greater rotational restriction per unit surface area (cm^2) of a solute when it is transferred from normal liquid water to the polarized water phase.) ΔE_{vp}, the *exclusion intensity* of water polarization is equal to the sum of the volume component, ΔE_v and the entropy component $1.23\Delta e^*$ of the polarization energy. $\mathcal{U}_{vp,}$ called either "exclusion intensity" or the volume component of the excess energy is equal to ΔE_{vp} multiplied by the molecular volume of water, 18.02 (cm^3). Similarly, $\mathcal{U}_s$, the "surface polarization energy" is equal to ΔE_s multiplied by 18.02 (cm^3.) R and T have the usual meaning. b, n and k are constants. b is a fractional number describing the low and steady probability of very large solute molecules in finding adsorbing sites on the water lattice. k and n are parameters describing the steepness of the declining probability of finding adsorbing sites in the water lattice with increasing molecular volume of the solute.

(2) Sensitivity of q-value to small changes in $\mathcal{U}_{vp}$

Elsewhere Ling has commented on this extreme sensitivity and likened it to that of a two-pan analytical balance which can measure accurately a sample a fraction of a milligram in weight on pans weighing 200,000 times heavier (Ling 1993.)

Physiological Chemistry and Physics and Medical NMR

INDEX

Volume 37, 2005