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The intranuclear environment

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Summary

Many of the Chapters in this volume are concerned with processes or structures inside the nucleus, and it is relevant to consider the properties of their environment, or rather of the multiple different and specific environments which must exist in local regions of the highly heterogeneous intranuclear space. Relatively little is known about the fundamental physical properties of these environments, and theoretical treatments of phenomena in such concentrated mixtures of charged macromolecules are complex and as yet poorly developed. Some of the phenomena which occur at the molecular level are unexpected and counter-intuitive for biologists, although well known to colloid and polymer scientists, for example the existence of short-range attractive forces between macromolecules or structures with like charges. As a

background for the Chapters which follow, we consider here some of the particular features of intranuclear environments, how they may influence processes and structures in the nucleus, and their implications for working with nuclei.

1. The macromolecular environment

The particular properties of the macromolecular environment within the nucleus are only now becoming recognised (***1-5***), and we believe that they are central to understanding molecular interactions, the formation of structures, and processes in the nucleus. Values for the global concentration of macromolecules within the nucleus, measured by several different approaches, range from 65 to 220 mg/mL (**Table 1**). At these concentrations, phenomena termed "macromolecular crowding" are observed (***6-9*** and references therein) which arise basically because the thermodynamic activities of macromolecules greatly exceed their concentrations, and which have important implications for processes within the nucleus.

Table 1 Concentration of macromolecules in the nucleus

	mg/mL	Method
NUCLEI		
Hepatocytes, rat	100[a]	Chemical assays
HeLa cells	96	Interference microscopy
Hepatocytes, human	165	Interference microscopy
Spermatocytes, *S. gregaria*	220	Interference microscopy
Salivary gland cells (polytene), *Drosophila*	65	Interference microscopy
Glial cells, human	150-180	Interference microscopy
NUCLEOPLASM		
Oocytes, *Xenopus*	106[b]	Interference microscopy
CHROMATIN		
Interphase chromosomes, human	75	Calculation
Nucleosomes (HeLa cells)	30-60	Fluorescence correlation spectroscopy
Heterochromatin, *Euglena* sp.	400	Quantitative STEM
NUCLEOLI		
Hepatocytes, rat	270[a]	Chemical assays
Mesothelial cells, newt	220	Interference microscopy
HeLa cells	200	Interference microscopy
Oocytes, *Xenopus* (dense fibrillar region)	215[b]	Interference microscopy

[a]isolated nuclei and nucleoli; [b]as protein, all other values are totals.
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1.1. Reaction kinetics

Macromolecular crowding has important consequences for the thermodynamics of the cell ***(6-9)***, strongly affecting reaction kinetics ***(8,9)*** and diffusion processes ***(10)***. The thermodynamics of solutions with a high macromolecular content is specifically affected by

entropic effects termed "depletion forces" ***(11)***. These occur in a mixture of molecules of different sizes because contact between larger molecules (or particles) is favoured since it causes the excluded volumes which surround them to overlap, thus increasing the volume accessible to smaller molecules and increasing the system's entropy (***12, 13***). Particular local entropic environments may surround different macromolecules and structures (***14)***.

Crowding results in quantitative effects on both the rates and the equilibrium of reactions involving macromolecules ***(7-9,12,13,15)***; the changes depend upon the sizes of the molecules, on the crowding agents, and on the milieu ***(13,14)***. Enzymes which catalyze sequential reactions such as replication, repair and transcription in the nucleus form macromolecular complexes so that the product of one enzyme does not have to diffuse to reach another enzyme, thus increasing metabolic efficiency ***(16)***.

Relatively little is known about the fundamental physical properties of the intranuclear environment ***(17)***. In particular, It remains inadequately understood how biochemical reactions in the nucleus and in other intracellular compartments differ from those in the test tube ***(9,18)***. The rate laws for chemical reactions occurring in intracellular environments with macromolecular crowding like the nucleus, characterized by heterogeneity and confinement which make chemicals diffuse anomalously, have been studied previously ***(9,19,20)***. Here we consider the validity of two central concepts of classical chemical kinetics in the cell nucleus: chemical equilibrium and the law of mass action.

1.2. Chemical equilibrium and the law of mass action

In ligand-receptor dynamics, the ligand (L) and receptor (R) associate and dissociate reversibly following the reaction scheme:

$$L + R \underset{k_{-1}}{\overset{k_1}{\Leftrightarrow}} C \qquad (1)$$

where k_1 and k_{-1} are the forward and backward rate constants, respectively.

A *chemical equilibrium* is achieved in any closed reaction system such as Eq. (1) as $t \to \infty$. In this state, there is no net activity which means that the chemical concentrations are not changing in time. From the thermodynamic point of view, the chemical equilibrium is reached when the forces driving the reaction in Eq. (1) are equal and opposite. According to the zeroth principle of thermodynamics, the change in the net Gibbs free energy of the ligand and receptor reaction is null ***(21)***. In physico-chemistry, thermodynamics studies changes in states and their stability and is only concerned with the initial and final states of chemical species in reactions.

Chemical equilibrium can be understood from a different perspective, that of chemical kinetics which focuses on understanding reaction mechanisms and the time scales of changes of chemical concentrations. In the domain of chemical kinetics, chemical equilibrium is achieved when the rates of the forward and backward reactions are equal and opposite. The rate of reaction in classical chemical kinetics obeys *the law of mass action,* which says that the rate of a reaction is proportional to the product of the concentrations of the reactants. Applying the law of mass action, the equilibrium concentrations for Eq. (1) obey the following conservation expression:

$$-k_1[L]\,(t \to \infty)[R](t \to \infty) + \quad {}_{-1}[C](t \to \infty) = 0, \qquad . \quad .$$

Backward rate

where the square brackets denote concentrations and ($t \to \infty$) their value at equilibrium.

We can now derive an expression for the equilibrium constant, K_{eq},

$$K_{\text{eq}} = \frac{k_{-1}}{k_1} = \frac{[L](t \to \infty)[R](t \to \infty)}{[C](t \to \infty)}, \qquad (3)$$

which depends only on the thermodynamics properties of the reacting system in Eq. (1) ***(22)***. It is important to emphasize that the forward and backward elementary reactions continue at chemical equilibrium although at this stage there is no measurable change in the reaction since the forward and backward rates are equal.

These principles are valid for most experimental conditions in standard laboratory practice, that is closed, constant-volume systems maintained at constant temperature and pressure. The reactions occur in a homogeneous environments, typically that of an ideal gas or a well-mixed liquid phase. Unfortunately, biochemical reactions rarely occur in these conditions *in vivo*, particularly in the nucleus. Therefore, what are the principles of reactions in the nucleus?

1.3. Chemical equilibria

During the 1960s and early 1970s, A.G. Ogston and T.C. Laurent carried out pioneering investigations on protein thermodynamics in polymer solutions with properties similar to those found in the nucleus ***(23,24)*** where, as in other intracellular compartments ***(25)***, macromolecules occupy a significant fraction of the total volume. As a consequence, they are spatially constrained on the microscopic level by force fields such as steric repulsion and attractive interactions which occur between them. These forces can be either specific, if they depend on the structure of the interacting molecules, or non-specific if they depend on the global properties of the solvent or reaction medium. If the molecules are considered part of a mixture of hard

spheres, the total free energy of interaction between a specific molecule and all the other molecules in the crowded environment is inversely proportional to the probability of placing the specific molecule at a random location within the crowded medium ***(26)***. The total free energy depends then upon the numbers, sizes and shapes of the other molecules present in the reaction compartment ***(7,27)***. The effect of steric-repulsive forces on the volume available to a given molecule depends on the centre of mass of the molecule and the molecules already present in the solution or "background" molecules. If the molecule to be introduced into the reaction is much smaller than the background molecules, the available reaction volume is large as small molecules can diffuse between the large molecules. However, if the molecule introduced into the reaction has a similar size to the background molecules the available volume is substantially smaller, as the centre of a molecule can approach the centre of another only to the distance at which the surfaces of the molecules contact each other. This phenomenon is known as the volume exclusion ***(8)*** or depletion effect ***(11)***.

Allen P. Minton has systematically analysed the effects of background macromolecules on biochemical equilibria ***(13,27,28)***. By analogy, Minton treated the interstitial elements of free volume due to background molecules as pores. When the size of a pore is not much larger than that of an enclosed molecule, steric-repulsive interactions between the molecule and the pore boundaries result in a reduction of the volume available to it. Therefore, free energy is required to transfer the molecule from an element of unbounded solution into a pore of equal volume ***(29)***. In this case, the magnitude of excess work depends strongly upon the relative sizes and shapes of both the confined molecules and pores ***(30)***; this phenomenon is known as macromolecular confinement ***(7,30)***. Macromolecular crowding can also cause a different type of phenomenon: if the molecule bears a net charge opposite to that of the background molecules, then the molecule can be reversibly and nonspecifically adsorbed onto the surface ***(31)***. This is known as macromolecular adsorption ***(7)***. When the absorption is spontaneous, the free energy change is negative and its magnitude depends on factors which vary with the size and shape of the molecule ***(32)***.

The influence of macromolecular crowding on a chemical equilibrium is represented by an apparent equilibrium constant:

$$\tilde{K}_{\mathrm{eq}} = \Gamma K_{\mathrm{eq}}, \qquad (4)$$

where K_{eq} is the equilibrium constant measured in an ideal solution (in the case of the reaction in Eq. (1), this is Eq. (3)) and Γ is a nonideal correction factor. The activity coefficient is equal to unity if the equilibrium occurs in an ideal solution. Otherwise, the correction factor can take

constant values, different from unity. The value of the correction factor can be calculated theoretically from the hard spherical particle model in porous media, which assumes that the law of mass action is valid and the rate of the reaction is also subject to a non-ideal correction. The value of the correction factor depends of the sizes, shapes and concentrations of the reacting and background molecules ***(13)***. This model is valid under a restricted set of conditions: the rate of encounter between the ligand and receptor is larger than the rate of dissociation. In this case, macromolecular crowding does not occupy a significant fraction of the total volume and reactions are not subject to limited diffusion.

If macromolecular crowding occupies a significant fraction of the total volume, reactions will be subject to a limited and anomalous diffusion ***(10,33)***. The rate of encounter of reactants generally varies relative to the rate with which their intermediates break down. Under this condition, the law of mass action is invalid ***(9,19)*** and the classical picture of thermodynamic equilibrium also breaks down ***(34,35)***. The chemical equilibrium is not generally a true thermodynamic equilibrium, but rather a non-equilibrium steady state as $t \to \infty$. The exact value of this non-equilibrium steady state has to be calculated using kinetics approaches taking into account dynamics effects. The overall Gibbs free energy principle cannot be postulated *a priori* to determine the chemical equilibrium.

1.4. The law of mass action in the nucleus

The structural organisation of the nucleus is far from the homogeneous, well mixed solution typical of an *in vitro* experiment. The consequences of the complexities of the rates of reactions are only now becoming more generally understood ***(9,19,20,36)*** with the aid of various computational frameworks to extract rate laws or empirical rate equations from simulation experiments. Among these approaches, simulations based on lattice-gas automata are the most popular and widely used.

A systematic computational investigation of reactions in environments with a fraction of the total volume occupied by obstacles has revealed that there are at least two reaction rate laws governing reactions in heterogeneous media ***(19)***. For the bimolecular irreversible reaction scheme:

$$A + B \xrightarrow{f} C , \quad (5)$$

the rate of product formation, v, is governed by the following expression:

$$v = f\ a(t)b(t) , \quad (6)$$

where f is a rate function, and $a(t)$ and $b(t)$ are the reactant concentrations at time t. Note that if the law of mass action is valid, f is a rate constant.

The reaction kinetics of Eq. (5) are not determined by the nature of the background molecules, but by the value of the reaction probability between *A* and *B* and their initial concentrations. The computational analysis of reaction Eq. (5) has shown that the law of mass action is valid if the reaction probability between *A* and *B* is small and one of these chemical species is at a low concentration relative to the other.

In agreement with theoretical and experimental evidence of reactions occurring in heterogeneous media, the computational analysis of the elementary reaction in Eq. (5) shows that the rate law always follows time-dependent power-law behaviour as $t \to \infty$, known as fractal-like kinetics ***(37)***, in confined environments with high macromolecular content. Hence, the rate function follows:

$$f = k(t=1)\, t^{-h} \qquad t \geq 1\,, \tag{7}$$

where *k* is a rate constant and *h* is a constant measuring the dimensionally of the confined system. This constant is bounded between 0 and 1 ($0 \leq h \leq 1$). The results of computational investigations show that physically dissimilar structures of the cell exhibit the same type of rate laws. However, it is possible that complex covalent interactions of molecules with the molecular background can affect the rate laws. The replacement of the rate constants by a time-dependent rate coefficient has unexpected consequences. Experiments with reaction mechanisms known to be elementary are easy to follow in time and have been used to test fractal-like kinetics ***(37)***. However, the more complex reaction mechanisms of biochemical reactions are more difficult to follow in time ***(38)***. There is an alternative rate law for diffusion-limited reactions, where the reaction rate is equal to the encounter rate between reacting species ***(13)***. The rate of encounter decreases exponentially with increasing concentrations of background molecules, and not due to specific interactions between the reacting and background molecules. Under these conditions, theory ***(39)*** suggests that the rate function is

$$f = k_0 \exp(-g\, m)\,, \tag{8}$$

where k_0 is the rate of the reaction (encounter rate) in ideal conditions, *g* is a function of the relative sizes and shapes of the reacting molecules, and *m* is the concentration of the background molecules.

1.5. Experimental observations and relevance to biochemistry in the nucleus

Thermodynamic and reaction kinetic models to study the effects of macromolecular crowding upon reactions have been developed during the last 20 years ***(9,13,19,30,32,36)***. These are based on mesoscopic-level models that take simplified representations of the reacting

molecules, their interactions, and effects of the background non-reacting molecules ***(36)***. These models provide simplifications for quantitative study of the chemical equilibrium of reactions in environments as complex and intrinsically variable as the nucleus. To date, these models have been successfully employed to predict and study effects of background molecules upon the chemical equilibrium and reaction rates of diverse molecules *in vitro* ***(6-8,28,40)***. These *in vitro* studies are a useful starting point to further our understanding of the reaction kinetics in the nucleus and other intracellular compartments. However, there has not been any application to reactions *in vivo*.

The intracellular environment is more complex and dynamic than a test tube experiment with high a macromolecular content. As we pointed out, the nucleus is a complex structure formed of multiple compartments with different micro-environments. For example, reacting molecules can encounter a region of extremely high concentration of DNA, or an area of high concentration of lipids and proteins in the nuclear membrane, or a region crowded with soluble macromolecules. It is possible that differences in the nature of the macromolecular crowding agents, the hydrodynamics of molecules, and the geometry of the micro-environment can affect the reaction dynamics. These effects are under investigation in theoretical and experimental studies.

In conclusion, the chemical equilibria and reaction kinetics in the nucleus are expected to be governed by anomalous rate laws due to macromolecular crowding in reaction environments. Reactants are spatially constrained in crowded environments on the microscopic level by force fields such as steric repulsion, and by non-specific attractive interactions which can occur between reacting and background molecules. Theory shows that the free energy of a reaction changes with the number, size and shapes of other molecules present in the reaction environments and that reaction kinetics are affected by the limited diffusion of reacting molecules. We have considered how biochemical reactions within the nucleus could differ from those in the test tube. We must acknowledge that the current theory represents a simplified picture of the nuclear environment, but during the last 20 years systematic *in vitro* experimental work where the composition of the reaction environment is changed has begun to emulate the *in vivo* environment and experimental techniques are currently being developed for monitoring reacting concentrations in time within individual cells ***(41)***. These methods, together with the theory discussed here, will help quantitative understanding of how much biochemical reactions within the nucleus differ from those in test tubes.

2. Effects of macromolecular crowding in the nucleus

2.1. Enhanced intermolecular association

In crowded media, macromolecular association constants are predicted to be as much as several orders of magnitude greater than in the dilute solutions commonly employed for studies *in vitro* (***6***). Fig. 1A illustrates such an effect observed experimentally for the association of two 70S ribosomal particles of *E. coli* to form a 100S particle. The short-range attractive forces which occur between macromolecules or structures with like charges are also enhanced in crowded media (***42-44***).

These effects may contribute to the self-organisation of the macromolecular complexes which form intranuclear structures and compartments such as the nucleolus and the different types of intranuclear bodies (***1***). Exogenous macromolecules introduced into the nucleus may also form regions of high local concentration or "foci" (reviewed in ***1***). The possible significance of crowding for the formation of nuclear aggregates of macromolecules in pathological conditions (*see* the Chapters by von Mikecz et al and by Iwahashi and Hagerman in this volume) remains to be examined.

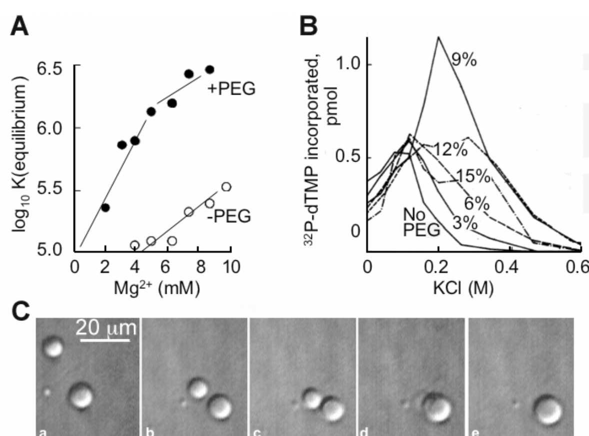

Fig. 1. Examples of effects of macromolecular crowding on macromolecular interactions. (**A**) The influence of crowding by poly(ethylene glycol) (PEG) (8 kDa, 4% w/v) on the equilbrium constant for the association of two *E. coli* 70S ribosome subunits to form a 100S particle. Note that in the presence of PEG, association proceeds in the absence of Mg^{2+} ions (reproduced from **(*46*)** by permission of Oxford University Press). (**B**) The influence of PEG (8 kDa) at different concentrations (w/v) on the response of the nick translation reaction of *E. coli* DNA polymerase I to the concentration of KCl (***45***) (Copyright (1987) National Academy of Sciences, USA, reproduced with permission). (**C**) Phase separation in a solution of deoxyhaemoglobin (Hb) and PEG (8 kDa, 1% w/v) in 0.15 *M* K phosphate buffer, pH 7.35. The dense spheres contain Hb at a concentration ~12 times higher than the solution, and were formed at 300 mg Hb/mL at 42ºC; similar phase separation was seen at 96 mg Hb/mL and 35ºC. The sequence shows droplets of the dense phase coalescing during a 55 sec period (***51***) (Copyright (2002) National Academy of Sciences, USA, reproduced with permission).

2.2. Modified reaction conditions

Crowding can greatly modify or extend the range of conditions under which enzymes or proteins are functional (***6***). For example in the presence of the crowding agent poly(ethylene glycol) (PEG), DNA polymerase I shows a different optimum concentration of KCl for nick translation (***45***) (Fig. 1B) and the requirement for Mg^{2+} ions for self-association of *E. coli* 70S ribosomal subunits is no longer seen (***46***) (Fig. 1A). The binding of *lac* repressor and RNA polymerase of *E. coli* to DNA *in vitro* is strongly dependent on salt concentration, whereas in the crowded intracellular environment *in vivo* their binding to specific sites is insensitive to the intracellular K^+ level (***47***). When determining the optimum conditions for a nuclear enzyme or reaction *in vitro*, the use of crowded conditions may therefore give a better indication of the characteristics of the reaction *in vivo*.

2.3. Compaction of extended polymers

Depletion forces in a crowded environment cause extended or linear polymers to collapse to more compact conformations (reviewed in ***5***). This effect could contribute to the compaction of extended macromolecular complexes in the nucleus, including the polynucleosome chains of interphase chromosomes.

2.4. Phase separation

In a solution of macromolecules or particles of different sizes and shapes, specific components may demix and form separate phases (e.g. ***49,49***). Phase separation can also be induced by the addition of counterions to a solution of macroions (***50***). In a concentrated solution of a single protein, phase separation can occur forming spherical regions containing the protein at a high concentration (e.g. ***51***) (Fig. 1C).

2.5. Effects on intrinsically unstructured proteins

Many nuclear proteins, including transcription factors and HMG proteins, contain regions of intrinsically disordered structure in solution (***52***). Disordered regions can become structured in crowded media (***53,54***), raising the possibility that in the nuclear environment these proteins may be more structured than predicted.

2.6. Stochastic nature of reactions

There is growing evidence that at least some reactions are stochastic in the nucleus. At the

molecular level, random fluctuations are inevitable when molecules are at low numbers per cell or collide with background molecules (***18***). In recent years, random fluctuations in the regulation of gene expression have been observed where small numbers of regulatory proteins interact with DNA binding sites in the gene's promoter region. These intrinsic noise effects have been measured recently using fluorescent probes (e.g. ***55, 56***). Low copy numbers of expressed RNAs may be significant for the regulation of downstream pathways (***57***). In this and other cases when there are only small numbers of molecules in the reaction volume, a stochastic modelling approach is required (***18*** and reference therein; ***36***). This means that experimentalists needs to shift from the deterministic kinetics of molecular concentration in a traditional reaction kinetics assay to a stochastic model, based on the probability that a molecular will be at a particular state.

3. The ionic environment

3.1. Cations

The concentrations of ions within the nucleus are often deduced from their measured global content in nuclei and used as a basis for preparing buffers for isolating and handling nuclei. However, these ions are unlikely to be distributed homogeneously in the intranuclear space because the surfaces of charged macromolecules and structures create particular local ionic environments, and theory predicts that their distributions in different regions are highly heterogeneous (e.g. ***58,59***). Simulation of the "interplay of electrostatics, dispersion forces, thermal motion, polarization, fluctuations, hydration, ion size effects and the impact of interfacial water structure makes it hard to identify a universal law" (***60***). The concept that "concentrations" of ions within the nucleus can be defined is therefore questionable.

The concentrations of ions within the nucleus may not be be reflected by those in buffers commonly employed to stabilise isolated nuclei because *in vivo*, the nucleus is stabilised by a further factor, the crowding effect exerted on it by the high concentration of macromolecules in the cytoplasm (***10***) which is relaxed when the cell membrane is removed. When this effect is replaced by a crowding agent such as PEG or dextran included in the cell lysis buffer, cations are no longer required in buffers to prepare stable nuclei (***5***; R. Hancock, unpublished data). The use of buffers containing a crowding agent for isolation of nuclei and intranuclear structures such as nucleoli, instead of conventional cation-containing media, may therefore reproduce more closely the conditions *in vivo*.

3.2. Hydrogen ions

The concentration of hydrogen ions measured by pH-sensitive dyes in the nucleus of a number of cell types is ~7.3, and higher by 0.3-0.5 pH units than that in the cytoplasm (***61,62***). The local concentration of hydrogen ions, like that of other cations, is expected to vary considerably near the surfaces of macromolecules and structures; for example, localised regions of pH 4.5 are predicted to occur in the minor groove of DNA in a buffer at pH 7.5 (***58***).

Spitzer and Poolman (***63***), considering the cytoplasm, drew attention to the possibility that electrochemical gradients resulting from heterogeneous micro-environments caused by the charged surfaces of macromolecules could be involved in the transport of charged low molecular weight molecules.

4. The redox environment

Nuclei contain glutathione (GSH) at an estimated concentration in the millimolar range (***64,65***); newer methods yield somewhat lower values (reviewed in ***66***). GSH is one of two systems which reduce protein thiols in the nucleus, the second being based on thioredoxin 1 (Trx1) (***67,68***). The maintenance of a reducing environment appears to be important for many nuclear activities; depletion of GSH impairs the transcriptional activation of heat shock genes (***69***), cysteine residues in several transcription factors must be reduced for activity (reviewed in ***70***), and reducing conditions are required for maximal activity of telomerase (***71***). Redox-sensitive motifs occur in the majority of cell cycle-associated proteins which function in the nucleus in the G1 phase (***70***). The reducing environment may also contribute to promoting repair of oxidative damage to DNA and to protecting oxidant-sensitive proteins from oxidation (***72***). During the isolation of nuclei, dislphide crosslinks may be formed between nuclear proteins (e.g. ***73***), probably as a result of vigourous aeration during homogenisation of tissues.

The nuclear GSH pool escapes from nuclei in aqueous buffers (***74***), and there is therefore a case for including a reducing agent such as glutathione or dithiothreitol (***75***) in solutions for isolating nuclei and their components in order to reproduce the redox conditions *in vivo*.

5. Diffusion in the nucleus

The observed diffusion constants of macromolecules in the nucleus are at first view unexpectedly high. Compared to those in aqueous solution they are similar for oligodeoxynucleotides (***76***), 3-5 times lower for dextrans of 500-750 kDa (***77***,***78***), and ~2-3 times lower for enhanced GFP (EGFP) and rod-shaped oligomers of EGFP (***79***). Within the nucleolus, however, the latter probes diffuse ~20-fold more slowly than elsewhere in the nucleus (***79***). The

relatively unhindered diffusion of macromolecules within the nucleus contrasts with the much more restricted diffusion of intranuclear compartments and chromosomes (***80,81***).

Crowding imposes constraints to diffusion which vary with the size, shape and chemical properties of the diffusing molecules (***77,82***) and anomalous diffusion is observed in crowded environments (***83,84***). Diffusion rates may be higher than predicted, because depletion effects at the surface of a moving macromolecule produce a low-viscosity layer around it which strongly reduces the friction which it experiences (***85,86***).

Perspectives

The view of the nucleus as a crowded and confined mixture of charged macromolecules provides new perspectives for both experimental and theoretical approaches. In experimental studies, an oversimplified approximation to conditions *in vivo* would be provided by using buffers which are crowded by addition of PEG, dextran, or Ficoll (e.g. ***46***). These crowded solutions are presently the best systems to study nuclear enzymes, enzyme systems, and intranuclear structures such as the nucleolus *in vitro*. Limits on the salt content of buffers which will reproduce the environment *in vivo* are imposed by the exquisite sensitivity to salts of clustering and phase separation of proteins in concentrated solutions (e.g. ***87,88***).

Theoretical approaches of a new and more quantitiative nature are needed to understand the entropic and depletion effects. These forces are likely to be major contributors to the formation of structures, and are also crucial for modelling complex sequential processes such as replication and transcription (***3***) and for signalling and control networks. It should be noted that analogous problems arise in physical chemistry (colloid and interface sciences) and are currently investigated using both theoretical and experimental methods. Some of these studies can provide interesting clues to understand biophysical-chemical processes in the nucleus; for example, they bring new perspectives on the forces which may determine the conformation of interphase chromosomes (***5***). Interdisciplinary approaches associating nuclear biologists with mathematicians, colloid, surface and interface scientists are likely to be essential to understand the nucleus and the effects of macrolecular crowding in intracellular environments.

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References

1. Hancock, R. (2004) A role for macromolecular crowding effects in the assembly and function of compartments in the nucleus. *J. Struct. Biol.* **146,** 281-290.
2. Misteli, T. (2005) Concepts in nuclear architecture. *Bioessays* **27,** 477-487.
3. Marenduzzo, D., Micheletti, C., and Cook, P.R. (2006) Entropy-driven genome organization. *Biophys. J.* **90,** 3712-3721.
4. Iborra, F.J. (2007) Can visco-elastic phase separation, macromolecular crowding and colloidal physics explain nuclear organisation? *Theor. Biol. Med. Model.* **4**, 15.
5. Hancock, R. (2007) Packing of the polynucleosome chain in interphase chromosomes: Evidence for a contribution of macromolecular crowding. *Sem. Cell Dev. Biol.* (in press).
6. Zimmerman, S.B. and Minton, A.P. (1993) Macromolecular crowding: Biochemical, biophysical and physiological consequences. *Annu. Rev. Biophys. Biomol. Struct.* **22,** 27-65.
7. Minton, A.P. (2006) How can biochemical reactions within cells differ from those in test tubes? *J Cell Sci.* **119,** 2863-2869.
8. Minton, A.P. (2001) The influence of macromolecular crowding and macromolecular confinement on biochemical reactions in physiological media. *J. Biol. Chem.* **276,** 10577-10580.
9. Schnell, S. and Turner, T.E. (2004) Reaction kinetics in intracellular environments with macromolecular crowding: simulations and rate laws. *Prog. Biophys Mol. Biol.* **85,** 235-260.
10. Luby-Phelps, K. (2000) Cytoarchitecture and physical properties of cytoplasm: Volume, viscosity. diffusion, intracellular surface area. *Int. Rev. Cytol.* **192**, 189-221.
11. Asakura, S. and Oosawa, F. (1958) Interaction between particles suspended in solutions of macromolecules. *J. Polymer Sci.* **33,** 183-192.
12. Minton, A.P. (1997) Influence of excluded volume upon macromolecular structure and associations in 'crowded' media. *Cur. Op. Biotechnol.* **8,** 65-69.
13. Minton, A.P. (1981) Excluded volume as a determinant of macromolecular structure and reactivity. *Biopolymers* **20,** 2093-2120.
14. Lago, S., Cuetos, A., Martinez-Haya, B. and Rull, L.F. (2004) Crowding effects in binary mixtures of rod-like and spherical particles. *J. Mol. Recognit.* **17**, 417–425.
15. Ellis, R.J. (2001) Macromolecular crowding: obvious but underappreciated. *Trends Biochem. Sci.* **26,** 597-604.

16. Cavalier-Smith, T. (1987) The origin of eukaryote and archaebacterial cells. *Ann. NY Acad.f Sci.* **503,** 17-54.
17. Handwerger, K.E. and Gall, J.G. (2006) Subnuclear organelles: new insights into form and function. *Trends Cell Biol.* **16,** 19-26.
18. Turner, T.E., Schnell, S., and Burrage, K. (2004) Stochastic approaches for modelling in vivo reactions. *Comput. Biol. Chem*. **28,** 165-178.
19. Grima, R. and Schnell, S. (2006) A systematic investigation of the rate laws valid in intracellular environments. *Biophys. Chem.* **124,** 1-10.
20. Grima, R. and Schnell, S. (2006) How reaction kinetics with time-dependent rate coefficients differs from generalized mass action. *Chemphyschem* **7,** 1422-1424.
21. Hammes, G.G. (2000) *Thermodynamics and kinetics for the biological sciences*. Wiley-Interscience, New York.
22. Chandler, D. (1987) *Introduction to Modern Statistical Mechanics*. Oxford University Press, New York.
23. Laurent, T.C. and Ogston, A.G. (1963) Interaction between polysaccharides and other macromolecules. 4. Osmotic pressure of mixtures of serum albumin and hyaluronic acid. *Biochem. J.* **89,** 249-253.
24. Laurent, T.C. (1995) An early look at macromolecular crowding. *Biophys. Chem.* **57,** 7-14.
25. Fulton, A.B. (1982) How crowded is the cytoplasm? *Cell* **30,** 345-347.
26. Lebowitz, J.L., Helfand, E., and Praestga.E. (1965) Scaled particle theory of fluid mixtures. *J. Chem. Phys.* **43,** 774-779.
27. Minton, A.P. (1998) Molecular crowding: Analysis of effects of high concentrations of inert cosolutes on biochemical equilibria and rates in terms of volume exclusion. *Meth. Enz.* **295,** 127-149.
28. Minton, A.P. (1983) The effect of volume occupancy upon the thermodynamic activity of proteins: some biochemical consequences. *Mol. Cell. Biochem.* **55,** 119-140.
29. Giddings, J.C., Kucera, E., Russell, C.P., and Myers, M.N. (1968) Statistical theory for equilibrium distribution of rigid molecules in inert porous networks. Exclusion chromatography. *J. Phys. Chem*. **72,** 4397-4408.
30. Minton, A.P. (1992) Confinement as a determinant of macromolecular structure and reactivity. *Biophys. J.* **63,** 1090-1100.
31. Lakatos, S. and Minton, A.P. (1991) Interactions between globular proteins and F-actin in isotonic saline solution. *J. Biol. Chem.* **266,** 18707-18713.

32. Minton, A.P. (1995) Confinement as a determinant of macromolecular structure and reactivity. II. Effects of weakly attractive interactions between confined macrosolutes and confining structures. *Biophys. J.* **68,** 1311-1322.
33. Saxton, M.J. (1994) Anomalous diffusion due to obstacles: A Monte Carlo study. *Biophys. J.* **66,** 394-401.
34. Voituriez, R., Moreau, M., and Oshanin, G. (2005) Reversible diffusion-limited reactions: "Chemical Equilibrium" state and the Law of Mass Action revisited. *Europhys. Lett.* **69,** 177-183.
35. Voituriez, R., Moreau, M., and Oshanin, G. (2005) Corrections to the law of mass action and properties of the asymptotic t=infinity state for reversible diffusion-limited reactions. *J. Chem. Phys*. **122**.
36. Grima, R. and Schnell, S. (2007) A mesoscopic simulation approach for modeling intracellular reactions. *J. Stat. Phys.* **128,** 139-164.
37. Kopelman, R. (1988) Fractal reaction kinetics. *Science* **241,** 1620-1626.
38. Schnell, S. and Maini, P.K. (2003) A century of enzyme kinetics: Reliability of the K_M and v_{max} estimates. *Comm. on Theor. Biol.* **8,** 169-187.
39. Minton, A.P. and Ross, P.D. (1978) Concentration dependence of the diffusion coefficient of hemoglobin. *J. Phys. Chem.* **82,** 1934-1938.
40. Hall, D. and Minton, A.P. (2003) Macromolecular crowding: qualitative and semiquantitative successes, quantitative challenges. *Biochim. Biophys. Acta* **1649,** 127-139.
41. Srividhya, J., Schnell, S., Crampin, E.J., and McSharry, P.E. (2007) Reconstructing biochemical pathways from time course data. *Proteomics* **7,** 828-838.
42. Bhuiyan, L.B., Vlachy, V. and Outhwaite, C.W. (2002) Understanding polyelectrolyte solutions: macroion condensation with emphasis on the presence of neutral co-solutes. *Int. Rev. Phys. Chem*. **21**, 1-36.
43. Rescic, J., Vlachy, V., Outhwaite, C.W., Bhuiyan, L.B. and Mukherjee, A. K. (1999) A Monte Carlo simulation and symmetric Poisson–Boltzmann study of a four-component electrolyte mixture. *J. Chem. Phys*, **111**, 5514-5521.
44. Deserno, M., Arnold, A, and Holm, C. (2003) Attraction and Ionic Correlations between Charged Stiff Polyelectrolytes. *Macromolecules* **36***,* 249-259.
45. Zimmerman, S.B. and Harrison, B. (1987) Macromolecular crowding increases binding of DNA polymerase to DNA: An adaptive effect. *Proc. Natl. Acad. Sci. USA* **84**, 1871-1875.
46. Zimmerman, S.B. and Trach, S.O. (1988) Effects of macromolecular crowding on the association of E. coli ribosomal particles. *Nucleic Acids Res*. **16**, 6309-6326.

47. Richey, B., Cayley, D.S., Mossing, M.C., Kolka, C., Anderson, C.F., Farrar, T.C. and Record, M.T Jr. (1987) Variability of the Intracellular Ionic Environment of *Escherichia coli.* Differences between *in vitro* and *in vivo* effects of ion concentrations on protein-DNA interactions and gene expression. *J. Biol. Chem.* **262**, 7157-7164
48. Buitenhuis, J., Donselaar, L.N., Buining, P.A., Stroobants, A., and Lekkerkerker, H.N.W. (1995) Phase-separation of mixtures of colloidal boehmite rods and flexible polymer. *J. Coll. Interface Sci.* **175,** 46-56.
49. Frenkel D. (1999) Entropy-driven phase transitions. *Physica A* **263**, 26–38.
50. Linse, P. (2001) Structure and phase separation in solutions of like-charged colloidal particles. *Phil. Trans. Roy. Soc. Lond. A* **359**, 853-866.
51. Galkin, O., Chen, K., Nagel, R.L., Hirsch, E. and Vekilov, P.G. (2002) Liquid-liquid separation in solutions of normal and sickle cell hemoglobin. *Proc. Natl. Acad. Sci. USA* **99**, 8479-8483.
52. Xie, X., Vucetic, S., Iakoucheva, L.M., Oldfield, C.J., Dunker, A.K., Uversky, V.N. and Obradovic, Z. (2007) Functional Anthology of Intrinsic Disorder. 1. Biological Processes and Functions of Proteins with Long Disordered Regions. *J. Proteome Res.* **6**, 1882-1898.
53. Dedmon, M.M., Patel, C.N., Young, G.B. and Pielak, G.J. (2002) FlgM gains structure in living cells. *Proc. Natl. Acad. Sci. USA* **99**, 12681–12684.
54. Morar, A.S., Olteanu, A., Young, G.B. and Pielak, G.J. (2001) Solvent-induced collapse of alpha-synuclein and acid-denatured cytochrome c*. Protein Sci.* **10**, 2195–2199.
55. Elowitz, M.B., Levine, A.J., Siggia, E.D. and Swain, P.S. (2002) Stochastic gene expression in a single cell. *Science* **297**, 1183–1186.
56. Blake, W.J., Kærn, M., Cantor, C.R. and Collins, J.J. (2003) Noise in eukaryotic gene expression. *Nature* **422**, 633–637.
57. McAdams, H.H. and Arkin, A.P. (1997) Stochastic mechanisms in gene expression. *Proc. Natl. Acad. Sci. USA* **94**, 814–819.
58. Lamm, G and Pack, G.R. (1990) Acidic domains around nucleic acids. *Proc. Natl. Acad. Sci. USA* **87**, 9033-9036.
59. Beard, D. A. and Schlick, T. (2001) Modeling Salt-Mediated Electrostatics of Macromolecules: The Discrete Surface Charge Optimization Algorithm and Its Application to the Nucleosome. *Biopolymers* **58**, 106–115.
60. Koelsch, P., Viswanath, P., Motschmann, H., Shapovalov, V.L., Brezesinski, G., Mohwald, H., Horinek, D., Netz, R.R., Giewekemeyer, K., Salditt, T., Schollmeyer, H., von Klitzing, R.,

Daillant, J., and Guenoun, P. (2007) Specific ion effects in physicochemical and biological systems: Simulations, theory and experiments. *Coll. Surf. A* **303**, 110-136.

61. Dubbin, P.N., Cody, S.H. and Williams, D.A. (1993) Intracellular pH mapping with SNARF-1 and confocal microscopy. II: pH gradients within single cultured cells. *Micron* **24**, 581-586.
62. Seksek, O. and Bolard, J. (1996) Nuclear pH gradient in mammalian cells revealed by laser microspectrofluorimetry. *J. Cell Sci.* **109**, 257-262.
63. Spitzer, J. and Poolman, B. (2005) Electrochemical structure of the crowded cytoplasm. *Trends Biochem. Sci.* **30**, 536-541.
64. Soboll, S., Grundel, S., Harris, J., Kolb-Bachofen, V., Ketterer, B. and Sies, H. (1995) The content of glutathione and glutathione S-transferases and the glutathione peroxidase activity in rat liver nuclei determined by a non-aqueous technique of cell fractionation. *Biochem. J.* **311**, 889–894.
65. Bellomo, G., Palladini, G. and Vairetti. M. (1998) Intranuclear distribution, function and fate of glutathione and glutathione-S-conjugate in living rat hepatocytes studied by fluorescence microscopy. *Microsc. Res. Tech.* **36**, 243 - 252
66. Hansen, J.M., Go, Y-M. and Jones, D.P. (2006) Nuclear and mitochondrial compartmentation of oxidative stress and redox signaling. *Annu. Rev. Pharmacol. Toxicol.* **46**, 215-234,
67. Watson, W.H. and Jones, D.P. (2003) Oxidation of nuclear thioredoxin during oxidative stress. *FEBS Lett.* **543**, 144-147.
68. Halvey, P.J., Hansen, J.M., Johnson, J.M., Go, Y-M., Samali, A. and Jones, D.P. (2007) Selective Oxidative Stress in Cell Nuclei by Nuclear-Targeted D-Amino Acid Oxidase. *Antioxid. Redox Signal.* **9**, 807-816.
69. Rokutan, K., Hirakawa, T., Teshima, S., Honda, S. and Kishi, K. (1996) Glutathione Depletion Impairs Transcriptional Activation of Heat Shock Genes in Primary Cultures of Guinea Pig Gastric Mucosal Cells. *J. Clin. Invest.* **97**, 2242-2250.
70. Conour, J.E., Graham, W.V. and Gaskins, H.R. (2004) A combined in vitro/bioinformatic investigation of redox regulatory mechanisms governing cell cycle progression. *Physiol. Genomics* **18**, 196-205.
71. Borrás, C., Esteve, J.M., Viña, J.R., Sastre, J., Viña, J. and Pallardó, F.V. (2004) Glutathione Regulates Telomerase Activity in 3T3 Fibroblasts. *J. Biol. Chem.* **279**, 34332-34335.

72. Green, R.M., Graham, M., O'Donovan, M.R., Chipman, J.K. and Hodges, N.J. (2006) Subcellular compartmentalization of glutathione: Correlations with parameters of oxidative stress related to genotoxicity. *Mutagenesis* **21**, 383-390.
73. Kaufmann, S.H., Okret, S., Wikstrom, A.C., Gustafsson, J.A. and Shaper, J.H. (1986) Binding of the glucocorticoid receptor to the rat liver nuclear matrix. The role of disulfide bond formation. *J. Biol. Chem.* **261**, 11962-11967.
74. Loh, S.N., Dethlefsen, L.A., Newton, G.L., Aguilera, J.A. and Fahey, R.C. (1990) Nuclear thiols: technical limitations on the determination of endogenous nuclear glutathione and the potential importance of sulfhydryl proteins. *Radiat. Res.* **121**, 98–106.
75. Jackson, D.A., Yuan, J. and Cook, P.R. (1988) A gentle method for preparing cyto- and nucleo-skeletons and associated chromatin. *J. Cell Sci.* **90**, 365-378.
76. Politz, J.C., Browne, E.S., Wolf, D.E. and Pederson, T. (1998) Intranuclear diffusion and hybridization state of oligonucleotides measured by fluorescence correlation spectroscopy in living cells. *Proc. Natl. Acad. Sci. USA* **95**, 6043–6048.
77. Seksek, O., Biwersi, J., and Verkman, A.S. (1997) Translational diffusion of macromolecule-sized solutes in cytoplasm and nucleus. *J. Cell Biol.* **138,** 131-142.
78. Verkman, A. S. (2002) Solute and macromolecule diffusion in cellular aqueous compartments. *Trends Biochem. Sci.* **27**, 27–33.
79. Pack, C., Saito, K., Tamura, M. and Kinjo, M. (2006) Microenvironment and effect of energy depletion in the nucleus analyzed by multiple oligomeric EGFPs. *Biophys. J.* **91**, 3921-3936.
80. Tseng, Y., Lee, J.S.H., Kole, T.P., Jiang, I. and Wirtz, D. (2004) Micro-organization and visco-elasticity of the interphase nucleus revealed by particle nanotracking. *J. Cell Sci.* **117**, 2159-2167.
81. Görisch, S. M., Lichter, P. and Rippe, K. (2005) Mobility of multi-subunit complexes in the nucleus: accessibility and dynamics of chromatin subcompartments. *Histochem. Cell Biol.* **123**, 217–228.
82. Lukacs, G.L., Haggie, P., Seksek, O., Lechardeur, D., Freedman, N., and Verkman, A.S. (2000) Size-dependent DNA mobility in cytoplasm and nucleus. *J. Biol. Chem.* **275,** 1625-1629.
83. Weiss, M., Elsne, M., Kartberg, F. and Nilsson, T. (2004) Anomalous Subdiffusion Is a Measure for Cytoplasmic Crowding in Living Cells. *Biophys. J.* **87**, 3518-3524.
84. Banks, D.S. and Fradin, C. (2005) Anomalous Diffusion of Proteins Due to Molecular Crowding. *Biophys. J.* **89**, 2960-2971.

85. Koenderink G. H., Sacanna S., Aarts D. G. A. L. and Philipse A. P. (2004) Rotational and translational diffusion of fluorocarbon tracer spheres in semidilute xanthan solutions. *Phys. Rev. E* 69021804.
86. Tuinier, R., Dhont, J.K.G. and Fan, T.H. (2006) How depletion affects sphere motion through solutions containing macromolecules. *Europhys. Lett.* **75**, 929-935.
87. Broide, M.L., Tominc, T.M. and Saxowsky, M.D. (1996) Using phase transitions to investigate the effect of salts on protein interactions. *Phys. Rev. E* **53**, 6325-6335.
88. Stradner, A., Sedgwick, H., Cardinaux, F., Poon, W.C., Egelhaaf, S.U. and Schurtenberger, P. (2004) Equilibrium cluster formation in concentrated protein solutions and colloids. *Nature* **432**, 492-495.