ANALYSIS OF THE PENETRABLE SPACE WITHIN THE NUCLEUS

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SUMMARY
Radioactive glycogen molecules have been used as passive probes to compare cavity systems within nuclei and isolated chromatin. Isolated chromatin was found to possess a narrow range of microspaces with mean effective diameters between 4.0 and 4.5 nm (40 and 45 Å) depending on shape assumptions. Intact nuclei contained a far larger class of free spaces with average diameters in the order of 11.0-15.0 nm. This clearly shows that DNase-I (diam. 4.1 nm) can penetrate and occupy a large proportion of nuclear space even though this enzyme does not readily attack the undisturbed nuclear structure. A structure which simulated the pattern of penetrability and incorporated other known properties of chromatin was used to explain this DNase-I resistance of intact nuclei in terms of an ordered, compact, local structure interspersed by much larger spaces. A system for this local packing is suggested and the functional implications of this type of organization considered.

INTRODUCTION
The direct significance of nuclear cavity systems
When considering the structure of mammalian chromatin, access and transport questions arise that would be more easily assessed if there were quantitative studies of the size distribution of spaces and channels within the nucleus. Study of these spaces is effectively a study of the properties of the higher-order structure of chromatin and although chromatin structure in metaphase chromosomes, and at the sub-nucleosomal level is beginning to be understood (Bak, Zeuthen & Crick, 1977; Pardon et al. 1977; Finch et al. 1977) the nature of higher-order structures in resting nuclei is still unclear.

Information about nuclear spaces is also relevant to functional correlates of structural change, as in the many studies of spiralization and heterochromatin formation which link chromatin density, and by implication the spectrum of nuclear spaces, to the local synthetic activity of chromatin.

The method of study of the cavity systems: glycogen penetration
Natural glycogen has a very wide range of molecular weights which is easily modified by mild hydrolysis. It is uncharged, hydrophilic and highly deformable and its branched structure tends to preclude the formation of extensive, and thus orderly, secondarily bonded structures. Thus glycogen should passively occupy spaces in a rigid body in a manner determined by the effective molecular diameters of the various glycogen species and the effective molecular diameters of the spaces.
Nuclei and spherical granules of reprecipitated chromatin were exposed to $[^3H]$-glycogen solutions in order to compare the spectrum of molecular sizes of the penetrant glycogen with the spectrum of molecular sizes of the non-penetrant glycogen. This comparison was then used to calculate a size distribution of penetrable space within nuclei and chromatin.

The aim of this work

This paper reports the results of experiments which classify cavity systems in rat liver nuclei by their penetrable volume and relates these observations to nuclease studies and to the known shape of the nucleosome, allowing deductions to be made about the patterns of packing which produce these cavity systems.

It does not attempt a comprehensive description of the actual path of coiling of a chromosomal strand, but concerns itself with the ways the nucleosomes may pack against each other and some functional implications of this.

METHODS

Basic theory for interpretation of the penetrability of nuclei and chromatin by glycogen

The degree of penetration of a dilute solution of passive macromolecules into a system of cavities is partially determined by time and by the rate of diffusion, until the system has reached equilibrium. It then becomes determined by a simpler steric interference effect operating between penetrant molecules and the walls of cavities.

Preliminary studies of penetration by glycogen for 15 and 45 min demonstrated that time and rate of diffusion were not important factors under these experimental conditions, and that simple steric interference must be the major determinant.

Such results were expected since Sephadex beads, which possess far greater spherical radii than nuclei, have previously been shown to equilibrate very rapidly with solutions of macromolecules (Laurent & Killander, 1964; Hellsing, 1968).

Analysis of this type of steric interference effect, previously discussed by Ogston (1958), has been adapted to nuclear spaces by noting that the only portion of such a space which can freely equilibrate with a macromolecule is that portion more than one molecular radius from the walls of the cavity, classified here as penetrable space.

In these experiments penetrable space is expressed as a proportion of the total free-space which is, in turn, arbitrarily classified as the space penetrable by mannitol. Impenetrable space actually consists of 2 subclasses. One class is exemplified by very small spaces into which probing species cannot even enter, and the other comprises any space that is less than the molecular radius of the probe molecule from the walls of spaces which can be entered. This latter class of space is accessible to the surface of probe molecules but not penetrable by their centres. For spaces with simple shapes, penetration curves can be used to determine their radius directly. For example $(R_e-R_a)^N/R_a^N$ gives penetrable space as a proportion of total space where $N$ is 2 for open-ended cylinders, 3 for spherical holes, $R_e$ is the radius of the cavity and $R_a$ is the radius of the molecular probe.

Preparation of tritiated glycogen

Oyster glycogen was labelled by periodate oxidation followed by a $[^3H]$borohydride reduction, and finally by a further, thorough, non-radioactive borohydride reduction. It was freed from exchangeable tritium, salts, and charged macromolecular contaminants by either dialysis or desalting on ion-exchange resin followed by passage through beds of DEAE-, and CM-cellulose.

When lower-molecular-weight glycogen fragments were required, the glycogen was subjected to controlled hydrolysis in dilute HCl before periodate oxidation. The periodate oxida-
Nuclear structure

Tions were for 18 h at 37 °C under N₂ with final concentrations of 100 mg/ml glycogen and 10 mg/ml sodium periodate.

The [3H]glycogen was sorted into size-classes on Agarose or Sephadex columns, and these size-classes used to reconstitute a [3H]glycogen mixture with a suitable continuous spectrum of molecular weights over the range to be analysed. The final product had specific activities in the order of 5-10 x 10⁶ cpm/mg and is referred to as sorted glycogen.

Penetration of nuclei or reprecipitated chromatin by glycogen and mannitol probes

Polyamine-stabilized liver nuclei from 6 rats, or extracted and reprecipitated chromatin from 11 rats, were prepared as previously described (Wallace, Hewish, Venning & Burgoyne, 1971; Marshall & Burgoyne, 1976; Burgoyne & Mobbs, 1975) except that all sucrose was omitted from the final washing buffer.

A fresh pellet of nuclei or reprecipitated chromatin was dispersed in 1-6 ml containing 1-2 mg of sorted glycogen in buffer A, 0-1 mM EDTA and 6-25 mM [14C]mannitol (0-125 μCi of 14C). The nuclei or chromatin granules were incubated in this mixture for 45 min at 0 °C and then the suspended structures separated from the suspending medium as described below.

Centrifugal separation of nuclei and chromatin from the incubate containing glycogen probes

Low-speed centrifugation (10000 g for 5 min) was used to remove the bulk of the suspension fluid whilst minimizing centrifugal distortion of the spectrum of glycogen molecules. This was followed by a high-speed centrifugation (70000 g for 25 min) of the pellet under a bed of Dow-Corning 550 silicone oil which compressed the pellets and decreased the volume of the interstitial space. Insufficient care with these centrifugations caused centrifugal distortion of the distribution of higher molecular weights of glycogen resulting in the appearance, as an artifact, of a peak at the front of penetratograms.

Extraction of the glycogen and mannitol probes from the nuclei, chromatin and their supernatants

All steps were carried out at room temperature and quantitative recoveries were not important as long as the losses were non-selective ones with respect to mannitol and to the various size-classes in the glycogen.

Resuspended pellets and combined supernatants had carrier mannitol added and were then extracted with water-saturated phenol. The phenol and precipitate was then back-extracted with water. Care was taken to use short, low-speed centrifugations (12000 g at 11 °C for 20 min). The corresponding aqueous extracts from the pellet were combined, extracted with diethyl ether, and passed through small (0.2-0.4 ml) plugs of DEAE-acetate and Na⁺ CM-cellulose.

Determination of the size-distribution of glycogen on agarose or Sephadex G-50

Columns of LKB-ultragel-ACA-34 or Sephadex G-50 (fine), approximately 44 cm in length and of 1.8 cm internal diameter, were equilibrated with 4 mM EDTA (lithium salt), pH 7.4 containing 2 % ethylene glycol and 0.04 % Triton X-100. The loads were 1-5 ml and contained 4000-10000 cpm with respect to [14C]mannitol, plus a small amount of 14C sheared mouse DNA to act as a void volume marker for the fractionation.

After fractionation of both the penetrant and the supernatant mixtures the [14C]mannitol peaks were used to mark the column volume and also summed to provide a normalization constant for each run. The 3H count in each fraction was then divided by its corresponding normalization constant to give a normalized glycogen profile for the penetrant and supernatant mixture.

Finally, each normalized point on the profile of the penetrant glycogen was divided by the corresponding point from the profile of the normalized supernatant.

This final ratio was then plotted against the relative elution volume to produce a penetrato-
gram which showed the proportion (P) of free-space which any size-class glycogen could occupy without steric interference. In summary the 2 curves were normalized as follows:

\[
\frac{([\text{H}]\text{glycogen counts (x)} \text{in any molecular size-class recovered from the pellet})}{([\text{C}]\text{-mannitol counts (y)} \text{recovered from the pellet})} \text{... (A)}
\]

\[
\frac{([\text{H}]\text{glycogen counts (X) in the corresponding size-class recovered from supernatant})}{([\text{C}]\text{mannitol counts (Y) recovered from the supernatant})} \text{... (B)}
\]

Then the ratio \( P = \frac{A}{B} \) or \( P = \frac{x}{y} \times \frac{X}{Y} \) was routinely plotted against molecular size of glycogen as represented by the relative elution volume of the glycogen classes on a gel-filtration column (Figs. 1, 2). Relative elution limits of 0 and 100 % were defined by the elution volumes of [\text{C}]DNA and [\text{C}]mannitol respectively.

Microperoxidase, glucagon, myoglobin, haemoglobin and catalase were used as primary standards covering a range of equivalent molecular diameters over approximately 1·6, 1·9, 3·4, 5·3 and 9·1 nm respectively. When comparing nuclei to chromatin these points were used directly (e.g. Table 1, p. 7) but, when necessary, linear interpolations were made between them.

RESULTS

The nature of the chromatin precipitate used in penetration studies

Although some degradation must have occurred over the 20-h period of preparation it was acceptably low. The DNA in the granules was double-stranded, and approximately 3 nucleosomes in length as a result of the controlled endonucleolysis necessary for extraction. The granules contained all 5 histones as demonstrated by acrylamide gel electrophoresis (Panyim & Chalkley, 1969). The chromatin bodies were small, spherical bodies, or larger masses with spherical sectors on their surfaces as previously described (Burgoyne & Mobbs, 1975), and pellets of them had a lower total porosity with higher density than nuclei.

Comparison between 'penetratograms' from nuclei and reprecipitated chromatin

Each of the 4 penetratograms shown in Figs. 1 and 2 shows an analysis of distribution of penetrable space within packed pellets of nuclei and chromatin.

The vertical axis shows the proportion of space penetrated by mannitol which was penetrated by glycogen with the molecular diameters determined by the horizontal axis. The horizontal axis is, in effect, a non-linear scale of molecular diameters, and is based on the retention characteristics of Sephadex G-50 or Ultrogel ACA-34.

Penetration of nuclei and chromatin by glycogen probes in the range 1·2 to 3·5 nm.

Inspection of the penetratograms shown in Fig. 1 clearly demonstrates that the spaces throughout the chromatin granule sterically inhibit large molecular probes more than the cavity systems penetrating the intact nucleus.

The conclusions that can be drawn from these curves are interesting but limited in interpretation. Penetratograms show that nuclei contain spaces with an average, effective, diameter much larger than that found in the chromatin precipitate, but which is still sufficiently small or broad enough in range to be interfering significantly with the smallest classes of probe molecules used.

Penetration of nuclei and chromatin by glycogen probes in the diameter range 3·5 to 10·0 nm. Fig. 2, which shows penetratograms from nuclei and chromatin as resolved on a column of agarose, confirms that nuclei contain a larger class of spaces than the
Nuclear structure

Since the nuclear penetratogram still has a considerable slope in the region of 9.1 nm the nuclei must have a large proportion of its spaces comparable in size to 9.1-nm diameter particles but somewhat larger than them. Although a small number of extremely large cavities could give a similar degree of penetration, they could not give the observed slope. When the shape of the nuclear penetratogram was compared to that expected for various combinations of size and shape of space it best fitted a

Fig. 1. Penetratogram from a chromatin pellet compared with one from intact nuclei: analysis on Sephadex G-50. Reprecipitated chromatin bodies and nuclei were exposed to a mixture of [3H]glycogen molecules with a continuous size spectrum suitable for resolution on Sephadex G-50. Fractionation on Sephadex G-50 was used to compare the size-spectrum of penetrant glycogen to that of the solution they were in equilibrium with. Space penetrable by [14C]mannitol was defined as 100% of the intrapellet space and the proportion of this space that is penetrable by the various size classes of glycogen is shown as P on the vertical axis. The horizontal axis shows the relative elution volume of the particular size classes of glycogen. Zero on the horizontal axis corresponds to molecular diameters too large to penetrate Sephadex G-50 beads (i.e., which elute at Sephadex G-50 void volume), and 100% corresponds to molecular diameters so small as to be indistinguishable from mannitol. The vertical lines A, B, C show the relative elution volumes of the standard polypeptides, myoglobin, glucagon, and microperoxidase with the molecular diameters of 3.4, 1.9, and 1.65 nm, respectively. ▲, reprecipitated chromatin; ○, intact nuclei.
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mixed system in which a large proportion of spaces 11.0 to 15.0 nm wide dominates the pattern.

The penetratogram from chromatin in Fig. 2 is, as expected from Fig. 1, a curve characteristic of a narrow range of small spaces. The shape of this curve was compared to expectations for various combinations of size and shape of space and it best fitted a system of spaces with an effective diameter in the range 4.0 to 4.5 nm.

![Graph showing penetratogram](image)

Fig. 2. Penetratogram from a pellet of reprecipitated chromatin: analysis on Ultrogel ACA 34. All experimental details as for Fig. 1 except that LKB Ultrogel ACA 34 was used to analyse the glycogen size spectra and the original glycogen size spectrum was one that was suitable for resolution on ACA 34 gel. The vertical lines A, B, C show the relative elution volumes of the standard polypeptides, catalase, haemoglobin, myoglobin with the molecular diameters of 9.1, 5.3, and 3.4 nm, respectively. ▲, reprecipitated chromatin; ●, intact nuclei.

It should be noted that the errors in such calculations have 2 sources: assumption of space shape introduces an unavoidable uncertainty but the technical limitations of gel filtration with molecular diameter standards is probably the most serious source of error.

The penetratogram from chromatin in Fig. 2 also gave a very clear demonstration of how interstitial or interbody space was determined from penetratograms. The very long section between 5.3 nm and the origin, which was approximately level, demonstrated that the chromatin pellet contained approximately 7% penetrable space.
which had effective diameters greatly in excess of the maximum size of the glycogen probes used in these experiments. This is the expected behaviour of interstitial space. Finally a direct comparison of the 2 penetratograms in Fig. 2 indicates the possibility that small spaces like those in chromatin make a major, albeit partially obscured contribution to the penetratogram from nuclei. The results however do not prove this, they are only compatible with it. See also Table 1.

Table 1. Penetrable space classes in chromatin and nuclei

<table>
<thead>
<tr>
<th>% of total penetrable space in chromatin</th>
<th>in nuclei</th>
<th>Size class of penetrable space</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>6</td>
<td>Interstitial space between bodies causing constant vertical displacement of penetratograms</td>
</tr>
<tr>
<td>&lt;1</td>
<td>8</td>
<td>Large intrabody space that can equilibrate with 9-1-nm-diam. molecules</td>
</tr>
<tr>
<td>~1</td>
<td>17</td>
<td>Space that equilibrates with 5-3-nm-diam. molecules but not 9-1-nm-diam. molecules</td>
</tr>
<tr>
<td>4</td>
<td>11</td>
<td>Space that equilibrates with 3-4-nm-diam. molecules but not 5-3-nm-diam. molecules</td>
</tr>
<tr>
<td>87</td>
<td>58</td>
<td>Space that equilibrates with mannitol but not 3-4-nm-diam. molecules</td>
</tr>
</tbody>
</table>

Determination of total space penetrable by mannitol

Suspensions of nuclei or chromatin were exposed to solutions of buffered [14C]-mannitol and then centrifuged from the suspending medium by the procedure and under the conditions used for penetration studies involving glycogen. The nuclear pellets were found to have approximately 73% of their total volume penetrable by mannitol, and the reprecipitated chromatin had 57% of its volume penetrable by mannitol. Since interstitial volume in these pellets is approximately 6-7% these values can be corrected to approximately 65 and 50% for nuclei and chromatin respectively.

Discussion

Although these studies using glycogen probes do not themselves provide sufficient information to suggest nuclear packing patterns they become far more significant when considered with the known behaviour of the enzyme probes DNase-I and micrococcal nuclease.

Results from penetration by glycogen probes show that DNase-I, with molecular diameter approximately 4.1 nm, must be able to penetrate a very large proportion of nuclear space (Fig. 2) and yet very little nucleolysis is observed until the nuclear structure is disrupted (Burgoyne, Mobbs & Marshall, 1976). In contrast, the chromatin precipitate is a structure that molecules as big as DNase-I must have
great difficulty in penetrating. While it is possible that the DNase-I resistance of nuclei is partly a function of direct shielding of the bridge and nucleosomal DNA by bound H1 histone, the fragility of this protection indicates that it may be more due to structural inhibition of DNase-I penetration. If this is so and if the chromatin packing does reflect some features of nuclear structure then the nucleus may consist of a compact DNase-I-impermeable structure, like that in chromatin pellets, inter-penetrated by much larger cavities or channels. Further support is given to this proposition by the observation that micrococcal nuclease (mol. diam. approx. 3.4 nm) attacks nuclei in a way that is almost independent of higher-order structure (Burgoyne et al. 1976). This contrast between the behaviour of DNase-I and micrococcal nuclease might be expected if their target sites were within a structure like that of the chromatin precipitate, since the limit of penetrability of this precipitate is larger than the molecular diameter of micrococcal nuclease but is in the order of DNase-I (Fig. 2).

Although not applicable to the problems of nucleosomal coiling per se, these results do give useful information about the way nucleosomes interact over short distances and allow a tentative explanation of some patterns of nuclear activity.

A system is proposed in which, when nucleosomes pack compactly they touch each other, with their centres approximately 10 to 11 nm apart, placed at the corners of a regular cubic array. Such a structure would result in spaces, each of which would be surrounded by 8 nucleosomes. The DNA crossbridge and possibly the H1 histone, would be expected to lie within these small spaces or 'cages'. This simple quasi-crystalline model would explain the contrasting behaviour of DNase-I and micrococcal nuclease with respect to the bridge DNA. It also gives a satisfactory 1:1 ratio of crossbridges to 'cages' and makes a number of predictions which can be tested. However it is important to note that the postulated array is not a true crystalline lattice since there is a very wide range of nucleosomal orientations compatible with this system.

**Predictions and functional implications**

Fig. 3 shows the agreement between a simulated penetratogram based on a simple cubic system of packing, and the most significant region of the penetratogram obtained experimentally from chromatin.

Only the higher-molecular-weight segment of the spectrum is considered because very low-molecular-weight probes would be affected to a far greater extent by factors of sub-nucleosomal shape. Although the degree of agreement is satisfactory it should be noted that this does not prove the model, it is merely consistent with it.

This structure also suggests that the molecular size of nuclear proteins should give some indication of restrictions on their intranuclear site of operation. Proteins with molecular weights in excess of approximately 40000 Daltons would be restricted to moving within the coarse (11-0-15-0 nm) nuclear space and would be excluded from nuclear domains or zones that have the postulated compact packing. Thus, eukaryote RNA polymerases with molecular weights of 400000-500000 Daltons (Weaver, Blatti & Rutter, 1971; Chambon, 1975) are far too large to operate within such
compactly packed regions, and similar restrictions exist for the replication-associated mammalian DNA polymerase, with a molecular weight of 170000 (Wintersberger, 1977; Bollum, 1975), mammalian DNA-relaxing enzyme (mol.wt. 70000; Keller, 1975), and mammalian ligase (mol.wt. 95000; Lindahl & Edelman, 1968). The observed sensitivity of newly synthesized DNA to DNase-I (Seale, 1975; Burgoyne et al. 1976; Hewish, 1977) is in accord with this since it implies that DNA synthesis

Fig. 3. Simulated penetratogram compared to the penetratogram obtained from chromatin precipitate. This shows a simulated penetratogram (solid line), based on the simple cubic-packing system, compared to the most significant region of the penetratogram obtained from chromatin (broken line). The marker peptides were used to convert the horizontal axis on the observed penetratogram to an approximately linear molecular-diameter scale. The simulation assumed a simple diagonal cross-bridge and also assumed simple 10-6-nm spheres as packing units, to avoid undue assumptions. It is, of course, recognized that this is not the true shape but the errors caused by irregularity in the nucleosomal shape will become progressively less as the probing species increases in molecular diameter and the comparison is only concerned with the penetration of the larger probes.
is occurring after disruption of some compact, self-protective system. Moreover both transcription (Gottesfeld, Murphy & Bonner, 1975; Garel & Axel, 1976; Panet & Cedar, 1977) and DNA synthesis (De Roeper, Smith, Watt & Barry, 1977) have, independently, been suspected of occurring in some unusually open state of chromatin.

The existence of a compact quasi-crystalline state of chromatin would also provide another reason for the existence of large cavities within chromatin, because such a quasi-crystalline structure cannot undergo localized unpacking and repacking unless large free spaces are adjacent to the active regions. This may possibly be the major explanation for the many observations of swelling accompanying synthetic activity in nuclei (Gurdon & Woodland, 1968), and may explain why swelling appears to be a prerequisite for activity (De Roeper et al. 1977) rather than a consequence of it.

Thus, in summary, penetration studies involving glycogen, studies of nucleolysis, and consideration of the substructure of chromatin have been combined to suggest that nuclei may be usefully described as compact, quasi-crystalline structures interpenetrated by a system of large free spaces. This structure appears to explain why chromatin must swell as a prelude to synthetic activity and also why zones engaged in RNA and DNA synthesis should be particularly sensitive to nucleolysis.

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REFERENCES


Nuclear structure


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