A gentle method for preparing cyto- and nucleo-skeletons and associated chromatin

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Summary

We describe a method for permeabilizing and extracting cells that preserves both structure and function whilst allowing the cell derivatives to be handled freely. Cells are encapsulated in microbeads of agarose; the coat of agarose, which is freely permeable to small molecules, forms a protective layer around fragile cell constituents. Cells are then permeabilized by the non-ionic detergent Triton X-100 or antibody and complement in a buffer whose ionic composition mimics that of the cytoplasm. The resulting structures have been characterized morphologically (by immunofluorescence and electron microscopy) and biochemically. Lysis with Triton removes both cell and nuclear membranes, and extracts most of the cytoplasm to leave chromatin surrounded by cytoskeleton; nucleus and cytoplasm then become accessible to triphosphates, enzymes and antibodies. Lysis with complement permeabilizes the cell membrane but leaves the nuclear membrane intact; triphosphates and restriction enzymes, but not antibodies, can then enter both nucleus and cytoplasm. Both types of lysis yield preparations whose chromatin template remains essentially intact, and which is replicated and transcribed at rates close to, or greater than, those found *in vivo*. Treatment of complement-lysed cells with Triton reduces the very efficient DNA synthesis, implying that the nuclear membrane is involved, directly or indirectly, in replication.

Key words: method, cytoskeleton, nucleoskeleton, permeabilize, chromatin.

Introduction

The skeleton of the cell is so very fragile that it is broken by all but the gentlest fractionation procedure. For example, pelleting cells permeabilized by the non-ionic detergent Triton X-100 disrupts their cytoskeleton, which then becomes further disrupted on resuspension by pipetting. Cells are rarely lysed in buffers containing physiological concentrations of ions, since isolated nuclei and chromatin aggregate into an unworkable mess. Some procedures permeabilize cell membranes using electric fields, detergents or abnormal ion concentrations, without allowing too much cytoplasm to be lost (for reviews, see Miller *et al.* 1979; Knight & Scrutton, 1986; Otero & Carrasco, 1987; Beckers *et al.* 1987). Then cell remnants retain much of the original structure and can, in some cases, even repair the damaged membranes and resume growth; most importantly, the cell interior is accessible to small tracer molecules. Other methods involve treating cells growing on coverslips, so allowing transfer from one solution to another without too much shearing; such cytoskeletons are usually well-preserved but they cannot be handled too vigorously without fixation. Methods for isolating nuclei in bulk almost invariably involve swelling cells in hypotonic buffers, which activate nucleases and extract much of the active polymerases (Jackson & Cook, 1985*b*, 1986*b*,*c*). We therefore felt that there was a need for another method that permitted the gentle deconstruction of the cell and also preserved vital functions.

In developing a method, we have borne a number of considerations in mind. First, we wished to permeabilize both the cell and nuclear membrane so that all major compartments were accessible to tracers and probes (e.g. radiolabelled triphosphates, antibodies, enzymes and oligonucleotides). Second, we have attempted to use 'mild' conditions. Third, and most importantly, we have made the preservation of nuclear

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function our major priority.

Our approach is an extension of a method originally developed for isolating naked DNA from eukaryotic cells in a form that could be manipulated without breaking it (Cook, 1984). Structure is preserved by providing the fragile DNA with a protective coat of agarose, by first encapsulating cells in agarose microbeads. An aqueous phase containing living cells in molten agarose is homogenized with an immiscible phase of liquid paraffin. On cooling, suspended agarose droplets gel into microbeads of about 50 μm in diameter. Protein complexes as large as $1{\cdot}5\times10^8 M_r$, but not the very much larger skeletal elements or chromosomal DNA, can diffuse through the agarose. Therefore ionic detergents extract nearly all protein and RNA from encapsulated cells, leaving naked DNA, which is nevertheless completely protected from shear. Triton at a more physiological salt concentration extracts most cytoplasmic proteins and RNA but leaves encapsulated chromatin surrounded by insoluble cytoskeletal elements (Jackson & Cook, 1985*a*). Encapsulated material is completely protected from shear and can be transferred from one buffer to another simply by pelleting, yet it is also accessible to molecular probes.

We now have developed this approach further. Cells are encapsulated, regrown in microbeads and then both cell and nuclear membranes are disrupted using Triton. Lysis and subsequent washing take place in a buffer whose composition is approximately cytoplasmic. (For recent reviews, see Roos & Brown, 1985; Lev & Armstrong, 1975.) The buffer (pH 7·4) contains 22 mM-Na^+, 130 mM-K^+, 1 mM-Mg^{2+}, <0·3 μM free Ca^{2+}, 132 mM-Cl^-, 11 mM-phosphate, 1 mM-ATP. 1 mM-dithiothreitol is an optional addition. We chose a phosphate buffer to control pH to 7·4 and adjusted sodium, potassium and phosphate ions roughly to cellular levels. Levels of Cl^- are unphysiologically high. (*In vivo* proteins are the counterions.) Controlling free Mg^{2+} levels posed a special problem. Current concern in our laboratory is with both the structure of chromatin and the integrity of DNA, and in earlier experiments we used EDTA to chelate any Mg^{2+} that might activate nucleases; although this preserved DNA integrity, it also decondensed heterochromatin (Jackson & Cook, 1985*a*). We therefore decided to use a more physiological chelating agent, ATP. Using 1 mM-ATP and 1 mM-Mg^{2+} (levels roughly those found *in vivo*) we find there is sufficient free Mg^{2+} to preserve heterochromatin structure but insufficient (because it is nearly all complexed with ATP) to activate nucleases. Fortunately, this formulation enables most ATP-utilizing enzymes to function and ensures that contaminating free Ca^{2+} is held at about 0·3 μM, well below critical activating concentrations (Carafoli, 1987).

As we are also interested in the nuclear membrane, we developed a variant method that preserves its structure. We permeabilize specifically the cell membrane using cytolytic antibodies and complement (Reid, 1986), whilst leaving the nuclear membrane intact. Of course, this has the disadvantage that we use undefined reagents (i.e. whole serum).

Using Triton or cytolytic antibodies, we lysed encapsulated HeLa cells and removed soluble material by washing in this isotonic buffer. Then we characterized the residual structures: the morphology of the cytoskeleton and nucleus was well preserved, both nuclear and cytoplasmic compartments were accessible to proteins and the DNA remained intact, and was replicated and transcribed at rates close to those found *in vivo*.

Materials and methods

Cells

HeLa cells were grown as suspension cultures in minimal essential medium plus 5% newborn calf serum. In most experiments cells were grown for 18–24 h in [*methyl*-^{3}H]thymidine (0·05 μCi ml^{-1}; ≈60 Ci mmol^{-1}) to label their DNA uniformly. This enabled corrections to be made subsequently for any slight variations in cell numbers.

Cell encapsulation and lysis

Cells were encapsulated in 0·5% agarose as described by Cook (1984). Odd large beads that might block pipettes were removed by filtering a dilute solution through monofilament nylon filters of 150 μm mesh (R. Cadisch and Sons, London N3 2JW) using a Swinex filter. A variety of different lysis buffers was used. (1) Triton, pH 8·0. One vol. encapsulated cells in phosphate-buffered saline was mixed with 3 vol. ice-cold lysis mixture containing 0·5% Triton X-100, 100 mM-KCl, 50 mM-NaCl, 10 mM-Tris·HCl (pH 8·0), 1 mM-Na_2EDTA, 1 mM-dithiothreitol; after 15 min on ice they were washed three times in ice-cold 100 mM-KCl, 50 mM-NaCl, 10 mM-Tris·HCl (pH 8·0), 1 mM-Na_2EDTA and 1 mM-dithiothreitol (Jackson & Cook, 1985*a*). (2) Triton, pH 7·4. Encapsulated cells were washed in ice-cold pH 7·4 buffer, Triton X-100 was added to 0·5% and, after 15 min on ice, the cells were washed three times in cold pH 7·4 buffer. pH 7·4 buffer was made by adding 100 mM-KH_2PO_4 to 130 mM-KCl, 10 mM-Na_2HPO_4, 1 mM-$MgCl_2$, 1 mM-Na_2ATP (Sigma type II), 1 mM-dithiothreitol, to bring the pH to 7·4. As the acidity of the ATP varied from batch to batch, various amounts of KH_2PO_4 must be added, never exceeding 0·01 vol. and generally increasing K^+ to 130·8 mM and PO_4^{3-} to 11·6 mM. The free Ca^{2+} levels (kindly measured by Peter Griffiths, Physiology Department, Oxford) of this buffer were below 0·3 μM and can be clamped at 0·1 μM using 40 μM-EGTA. Note that many tissue-culture media contain millimolar quantities of Ca^{2+}, so that cells should be well washed if calcium levels are of concern. Note also that Ca^{2+} levels may rise transiently as cells are lysed, affecting cytoskeletal structure (Schliwa *et al.* 1981). (3) Complement, pH 7·4. Antiserum directed against HeLa cell surface antigens was obtained by injecting HeLa cells in phosphate-buffered saline subcutaneously into rabbits (3 injections at weekly intervals) and collecting the serum 1 week later. Encapsulated cells

were incubated with 0·5 vol. heat-inactivated whole antiserum for 30 min at 20°C, washed twice in pH 7·4 buffer and incubated for 10 min at 20°C with 0·1 vol. rabbit complement, which had a low toxicity for human lymphocytes (SeraLab), and then the lysed cells were washed three times in ice-cold pH 7·4 buffer. Note that serum, the source of complement, contains millimolar amounts of free Ca^{2+} and Mg^{2+} (as well as many other unknown factors) and that these ions are essential for lysis; therefore, the milieux during lysis by complement and by Triton in the pH 7·4 buffer are different. (4) Complement, pH 8·0. Cells were washed in 100 mM-KCl, 50 mM-NaCl, 10 mM-Tris·HCl (pH 8·0), 1 mM-Na_2EDTA and 1 mM-dithiothreitol, lysed by antibody and complement as described above and rewashed in the same buffer. (5) Triton, pH 8·0 and 2 M-NaCl. One vol. of encapsulated cells in phosphate-buffered saline was mixed with 3 vol. ice-cold lysis mixture that contained 0·5 % Triton X-100, 2·0 M-NaCl, 10 mM-Tris·HCl (pH 8·0), 100 mM-Na_2EDTA; after 15 min on ice they were washed three times in ice-cold 2·0 M-NaCl, 10 mM-Tris·HCl (pH 8·0), 1 mM-Na_2EDTA (Cook *et al.* 1976).

Light microscopy and immunofluorescence

Lysed encapsulated cells were generally washed once in the appropriate buffer minus dithiothreitol prior to incubations (45 min at 20°C or 2 h at 4°C) with primary antibodies, then washed three times with the same buffer, incubated (45 min at 20°C or 2 h at 4°C) with the appropriate second antibody, rewashed, viewed and photographed without fixation using a Leitz Orthoplan or a Zeiss Photomicroscope III fluorescence microscope fitted with the appropriate filters. A wide range of antibodies has been used including: (1) 414 (kindly provided by Dr L. I. Davis), a mouse monoclonal directed against a protein of $62\times10^3 M_r$ in the rat liver nuclear pore complex (Davis & Blobel, 1986). The supernatant from the cultured monoclonal cell line was used at a ratio of 1:1 and found to cross-react with the human antigen. Rhodamine-conjugated affinity-purified goat anti-mouse IgG (Cappel) diluted 1:25 with the appropriate buffer supplemented with 1 % bovine serum albumin, 5 % Triton was used to visualize the primary antibody. (2) Anti-Sm mouse monoclonal (Cappel), used (dilution 1 in 50) with thodamine-conjugated affinity-purified goat anti-mouse IgG (Cappel; dilution 1 in 100). A monoclonal directed against a glial fibrillar acidic protein (Amersham; dilution 1 in 50) served as a control first antibody. (3) Anti-human vimentin mouse monoclonal (Dako; dilution 1 in 50), used with fluorescein-conjugated affinity-purified rabbit anti-mouse IgG (dilution 1 in 100) on cells pre-fixed in 3 % formaldehyde in phosphate-buffered saline.

Lysed cells were also incubated with and without cytochalasin B (10 μg ml^{-1}, 30 min, 37°C), then with rhodamine-conjugated phalloidin (3 units ml^{-1}, 10 min, 4°C, Molecular Probes Inc) or 3,3′-dihexyloxacarbocyanine (1 μg ml^{-1}, 5 min, 20°C) and washed as above.

Macromolecular recoveries

Macromolecular recoveries were determined by measuring the amount of label associated with encapsulated cells before and after lysis and washing (Cook, 1984). Protein recoveries were determined using cells grown overnight in medium lacking leucine or methionine and supplemented with 2 % normal medium, 5 % dialysed foetal calf serum and L-[U-^{14}C]leucine (0·1 μCi ml^{-1}; 350 mCi mmol^{-1}) or L-[^{35}S]methionine (1 or 0·1 μCi ml^{-1}, 800 mCi mmol^{-1}). RNA was estimated using cells labelled in growth medium plus [5,6-^{3}H]uridine (53 Ci mmol^{-1}) for 2 min (50 μCi ml^{-1}) or 24 h (0·5 μCi ml^{-1}); in the former case cells were encapsulated prior to labelling and in the latter, values were corrected for incorporation (21 %) of uridine into DNA. DNA recoveries were determined by labelling for 24 h with [*methyl*-^{3}H]thymidine (47 Ci mmol^{-1}; 0·5 μCi ml^{-1}).

The protein content of extracted cells was analysed using 12 % to 20 % polyacrylamide gradient gels (see Fig. 4A). Cells, prelabelled with L-[^{35}S]methionine, were encapsulated ($2\cdot5\times10^6$ ml^{-1}), lysed and washed in buffers supplemented with 1 mM-phenylmethylsulphonyl fluoride and 25 μM-leupeptin, pepstatin and L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone, proteins dissolved in sodium dodecyl sulphate, ≈50 000 cts min^{-1} loaded per lane and subjected to electrophoresis and autoradiography (2 weeks). For Fig. 4B any soluble proteins remaining after washing were electroeluted from beads under isotonic conditions using slots in a 0·8 % agarose gel (4 h at 4 V cm^{-1}; buffer was recirculated to prevent pH drift). The buffering capacity of the pH 7·4 buffer was increased for this by increasing the phosphate ion concentration at the expense of the chloride ion so that the electrophoresis buffer contained 80·6 mM-KCl, 10 mM-Na_2HPO_4, 20 mM-K_2HPO_4, 9·4 mM-KH_2PO_4, 1 mM-$MgCl_2$, 1 mM-dithiothreitol and 1 mM-Na_2ATP. Beads were recovered, washed in pH 7·4 buffer, treated in various ways as described in the Fig. legend, rewashed in pH 7·4 buffer, samples were applied to a gel and autoradiographs (2 or 14 days) prepared as described above.

Electron microscopy

Beads were fixed in 2·5 % glutaraldehyde, post-fixed in 2 % osmium tetroxide, stained in 0·5 % uranyl acetate and embedded either in Epon–Araldite or the removable embedding compound diethylene glycol distearate (Capco *et al.* 1984), using the procedure described in detail by Fey *et al.* (1986). Samples were also critical-point dried, taking care to ensure samples were free of water (Ris, 1985).

Digestion with nucleases

Cells, prelabelled with [^{3}H]thymidine, were encapsulated (5×10^6 ml^{-1}), lysed, washed and incubated in an equal vol. buffer at 37°C for 0–80 min with 500 or 2500 units ml^{-1} *Eco*RI or 20 or 200 units ml^{-1} *Hae*III. In Fig. 5, units refer to the product of units ml^{-1} and time (h). After incubation, samples were removed and split. DNA fragment sizes were analysed in half the sample after addition of sodium dodecyl sulphate to 0·2 % by gel electrophoresis (0·7 V cm^{-1}; 16 h) through 0·8 % agarose in 40 mM-Tris·acetic acid (pH 8·3), 2 mM-EDTA and 20 mM-sodium acetate; after electrophoresis RNA was solubilized by soaking the gel in ribonuclease (20 μg ml^{-1}; 30 min, 20°C) and the gel was stained with ethidium and photographed. The other half was used to determine how much chromatin could be electroeluted from beads under isotonic conditions (using 0·8 % agarose gel and the buffer with extra phosphate described above, 15 h, 2 V cm^{-1}). The amount of ^{3}H remaining in beads was

determined by mixing 100-μl samples with 250 μl 2 % sodium dodecyl sulphate. More than 2 h later three 100-μl samples were spotted onto GF/C discs and the discs were extracted successively with 5 % trichloroacetic acid, ethanol and ether, then dried and their radioactivity was estimated using a Packard 300 CD scintillation counter.

Permeability of beads to DNA

The permeability of beads to DNA fragments of different sizes was determined by encapsulating bacteriophage λ DNA cut with *Hin*dIII, quickly washing in 100 vol. 50 mM-NaCl, 10 mM-Tris·HCl, 1 mM-EDTA in a microcentrifuge, then incubating the beads plus DNA at 20 °C and at various times determining the amount of DNA remaining in beads by quantitative densitometry after quickly washing beads, subjecting them to gel electrophoresis, staining with ethidium bromide and photography (Cook & Brazell, 1978).

Replication and transcription assays

General methods for assays have been described by Jackson & Cook (1985*b*, 1986*a*). Replication assays were conducted at 37 °C in appropriate buffers supplemented with 250 μM-dATP, dCTP and dGTP plus 125 μM-dTTP and 1 mCi ml^{-1} [^{32}P]dTTP ($\approx$3000 Ci mmol^{-1}) plus 100 μM-CTP, GTP and UTP plus 5 mM-potassium phosphate (pH 7·4) to ensure triphosphates were neutralized, plus 1 mM-ATP and 5 mM-$MgCl_2$. Transcription assays included 250 μM-CTP and GTP plus 125 μM-UTP and 1 mCi ml^{-1} [^{32}P]UTP ($\approx$3000 Ci mmol^{-1}) plus 1 mM-ATP plus 5 mM-$MgCl_2$. Initial rates were measured over the first 5 min. Encapsulated and lysed cells (5×10^6 ml^{-1} beads; $2{\cdot}5\times10^6$ ml^{-1} solution) and freshly made 10 times concentrated solution of supplements were incubated separately on ice, then at 37 °C for 5 min before mixing to start the reaction. Reactions were stopped by removing 100-μl samples and mixing them with 250 μl 2 % sodium dodecyl sulphate. More than 2 h later three 100-μl samples were spotted onto GF/C discs and the discs were extracted successively with 5 % trichloroacetic acid, ethanol and ether, then dried and their radioactivity was estimated using a Packard 300 CD scintillation counter.

Fluorometry

The ethidium-binding capacity of encapsulated and lysed cells was determined on ice-cold samples as described (Cook & Brazell, 1978; Jackson & Cook, 1985*a*). Every experiment involved a comparison of the fluorescence of γ-irradiated (9·6 J kg^{-1}) and non-irradiated samples. Encapsulated and lysed cells were incubated in pH 7·4 buffer at 37 °C and after various times EDTA, NaCl and ethidium bromide were added to final concentrations of 10 mM, 2 M and 8 μg ml^{-1}, respectively. Half were γ-irradiated and the fluorescence of both halves was measured. After subtraction of appropriate blanks, the fluorescence of dye bound to non-irradiated beads was divided by that bound to irradiated beads, effectively normalizing all non-irradiated values to those of equal amounts of fully relaxed DNA. This ratio reflects the proportion of nicked loops: at the ethidium bromide concentration used it is insensitive to changes in degree of supercoiling. To some extent it reflects the amount of RNA, which also binds ethidium, remaining after extraction with 2 M-NaCl and as this differs for samples lysed differently, relative changes occurring on incubation are more informative than absolute values.

Results

Morphology

Fig. 1A illustrates HeLa cells encapsulated in 0·5 % agarose microbeads. On resuspension in warm medium, they resume growth. Addition of Triton lyses cells and within seconds most soluble proteins diffuse out of beads (Cook, 1984), leaving a well-preserved nucleus surrounded by the Triton-insoluble cytoskeleton (Fig. 1B). (The various buffers are described by the lytic agent used (Triton or complement) and pH (8·0 or 7·4), the pH 8·0 buffer containing Tris and EDTA and the pH 7·4 buffer being closer to the physiological.) Cells lysed with Triton at pH 8·0 retain the major nuclear functions (Jackson & Cook, 1975*a*,*b*, 1986*a*,*b*); for example, they replicate DNA in a cell-cycle-dependent manner at a rate *in vitro* that is at least 85 % of the rate found *in vivo*. However, despite such excellent preservation of function, their structure is not so well-preserved, since some heterochromatin decondenses (see below). Therefore, we explored lysis with Triton and our pH 7·4 buffer that more closely mimics the ionic content of the living cell. The light microscope reveals an excellently preserved nucleus surrounded by cytoplasmic remnants (Fig. 1C). Lysis with complement rather than Triton swells the cells and induces some cytoplasmic loss (Fig. 1D). (HeLa are particularly resistant to lysis by complement, presumably because their cytoskeleton is so well developed. Other cells, for example lymphocytes and their derivative, are lysed more easily, with almost complete loss of cytoplasm.) This loss can be visualized after staining with 3,3′-dihexyloxacarbocyanine, a lipophilic and fluorescent dye (Terasaki *et al.* 1984); Triton extracts all membranes (cf. Fig. 1E and G), whereas complement lysis leaves the endoplasmic reticulum and the nuclear membranes essentially intact (cf. Fig. 1E and F).

Electron micrographs of typical conventional thin sections of these derivatives are illustrated in Fig. 2. (Note that heterochromatin of unlysed cells varies greatly and this is naturally reflected in lysed derivatives; we therefore present typical cases.) As expected, Triton removes both cell and nuclear membranes and extracts most cytoplasm (cf. Fig. 2B and C with A). In many cases the cytoskeleton contracts from the surrounding agarose coat. The improved buffer preserves nuclear structure slightly better than the pH 8·0 buffer that we used earlier; for example, there are more peripheral clumps of heterochromatin and typically the chromatin is less 'washed-out' (cf. Fig. 2B and C). Nevertheless, much heterochromatin has decon-

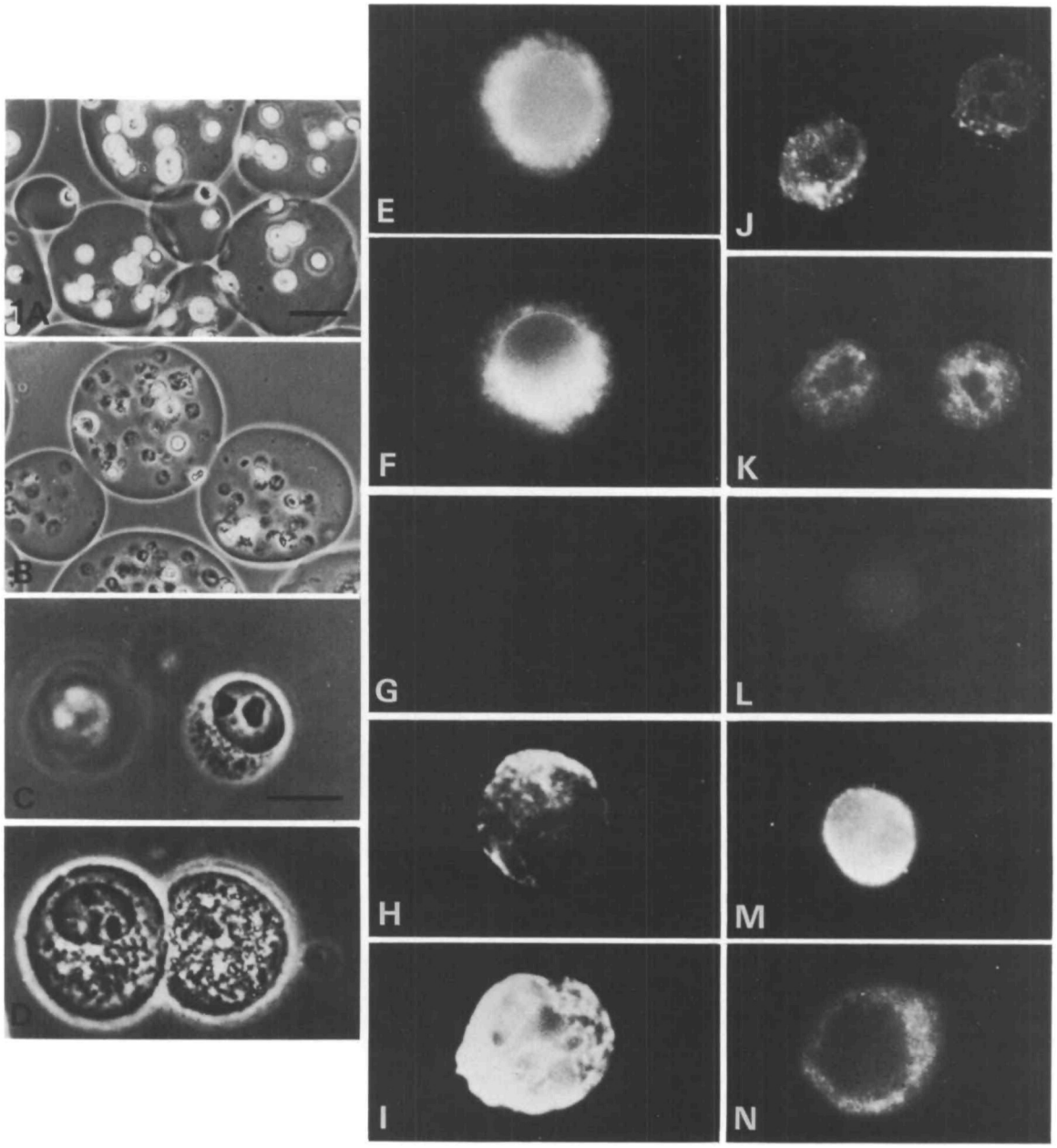

Fig. 1. The morphology of encapsulated HeLa cells and their derivatives lysed with Triton or complement. A–D. Phase-contrast micrographs: A, unlysed; B, lysed, Triton/pH 8·0; C, lysed, Triton/pH 7·4; D, lysed, complement/pH 7·4. E–G. Fluorescence micrographs using the lipid stain, 3,3′-dihexyloxacarbocyanine: E, unlysed; F, lysed, complement/pH 7·4; G, lysed, Triton/pH 7·4. H–I. Fluorescence micrographs using rhodamine-conjugated phalloidin: H, lysed, complement/pH 7·4; I, lysed, complement/pH 7·4 and then incubated with cytochalasin B. J–N. Immunofluorescence using various first antibodies: J, lysed, Triton/pH 7·4, anti-nuclear pore complex; K, lysed, Triton/pH 7·4, anti-Sm; L, lysed, Triton/pH 7·4, anti-glial protein (i.e. control); M, lysed, Triton/pH 7·4, fixed, anti-vimentin; N, lysed, complement/pH 7·4, fixed, anti-vimentin. A–B and C–N are at the same magnification, respectively. Bars: 50 and 10 μm.

densed. (As expected, spermine and spermidine lessen decondensation (results not shown) but probably have other effects.) Nuclear structure is excellently preserved on complement-mediated lysis (Fig. 2D). The holes punched by the attack complex of complement in the cell membrane are too small to be seen at this magnification, but in consequence the cytoplasm usually becomes vacuolar (this is not well illustrated in Fig. 2D; but see Fig. 3D). Our improved buffer also maintains the morphology of internal membranes, including the nuclear membranes, and those of mitochondria, organelles that are most sensitive to abnor-

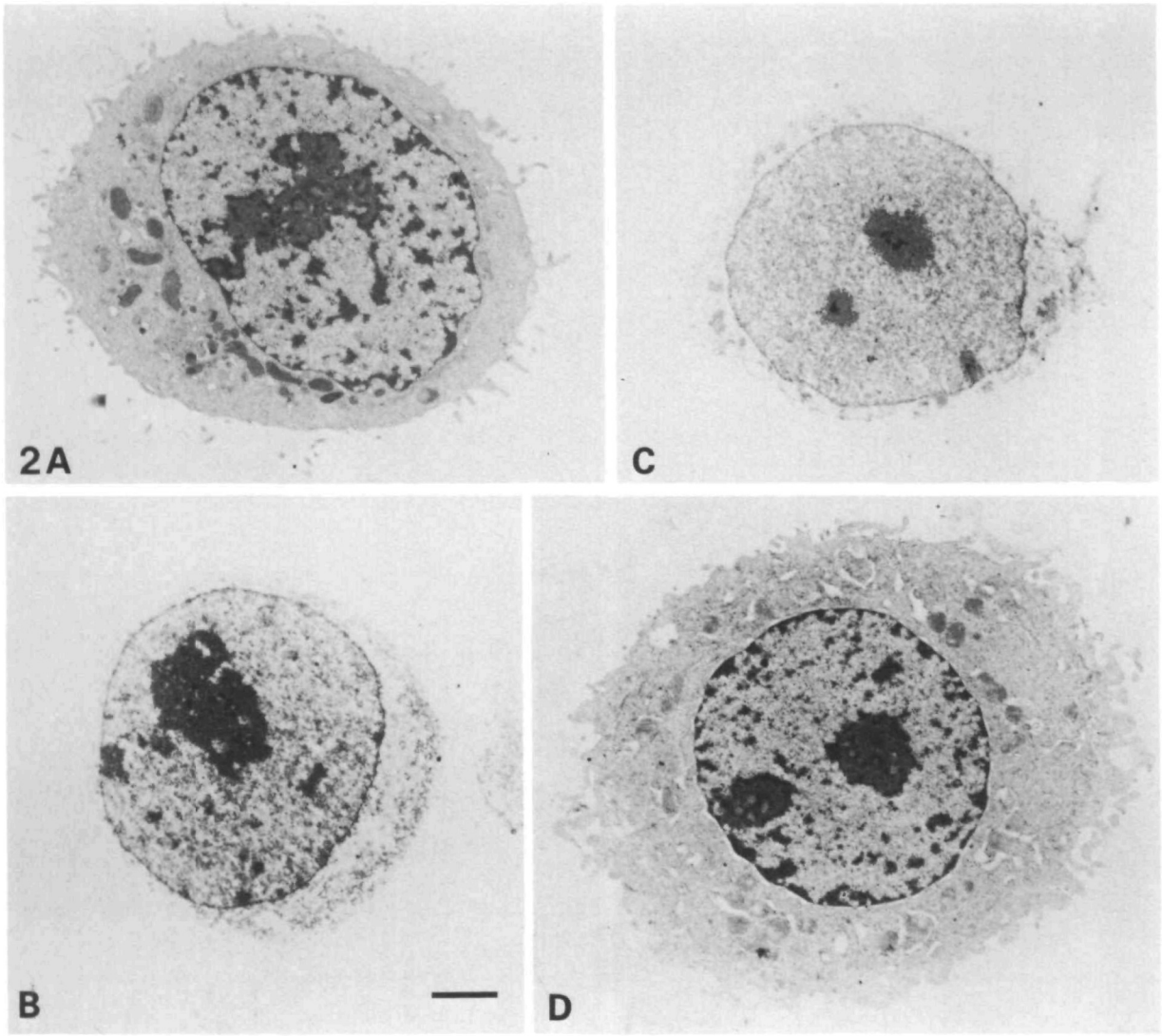

Fig. 2. Electron micrographs of thin sections of encapsulated HeLa cells. A. Unlysed; B, lysed, Triton/pH 7·4; C, lysed, Triton/pH 8; D, lysed, complement/pH 7·4. All are at the same magnification. Bar, 2·5 μm.

mal ionic concentrations.

Conventional sections used for electron microscopy are too thin (about 50 nm, with only the top few nm being accessible to stain) to reveal much detail of structures many μm long; therefore, skeletal structures are better seen in thicker (resinless) sections (Capco *et al.* 1984). Fig. 3 illustrates such 250 nm thick sections, where the surrounding filaments of agarose can be clearly seen. These thicker sections also give a better impression of the average density of material in extracted cells and appear quite different from thinner sections; for example, the peripheral clumps of heterochromatin become more difficult to see. On lysis in Triton (Fig. 3B and C) the cytoskeleton collapses away from the agarose, whereas complement vacuolates and swells the cytoplasm (Fig. 3D).

Whether skeletal networks like the microtrabecular lattice (Wolosewick & Porter, 1979) and nuclear matrix (Agutter & Richardson, 1980), which are visible in the electron microscope only after fixation or exposure to abnormal salt concentrations, also exist *in vivo* is controversial. (See, for example Ris, 1985.) Therefore, we have taken care to use fixation and drying procedures that minimize the artefactual creation of filamentous structures. Thus, we fixed freshly lysed cells in the (Ca^{2+}-free) pH 7·4 buffer with glutaraldehyde, conditions that best preserve nuclear morphology (Skaer & Whytock, 1977) and prevent ribonucleoprotein aggregating into filaments (Lothstein *et al.* 1985). We also critically point dried them prior to sectioning, using conditions that both permitted (Capco *et al.* 1984; Wolosewick & Porter, 1979) and prevented (Ris, 1985) subsequent visualization of a microtrabecular lattice; at the magnification used in Fig. 3 no gross differences could be seen, but at higher magnifications the different methods yielded marked

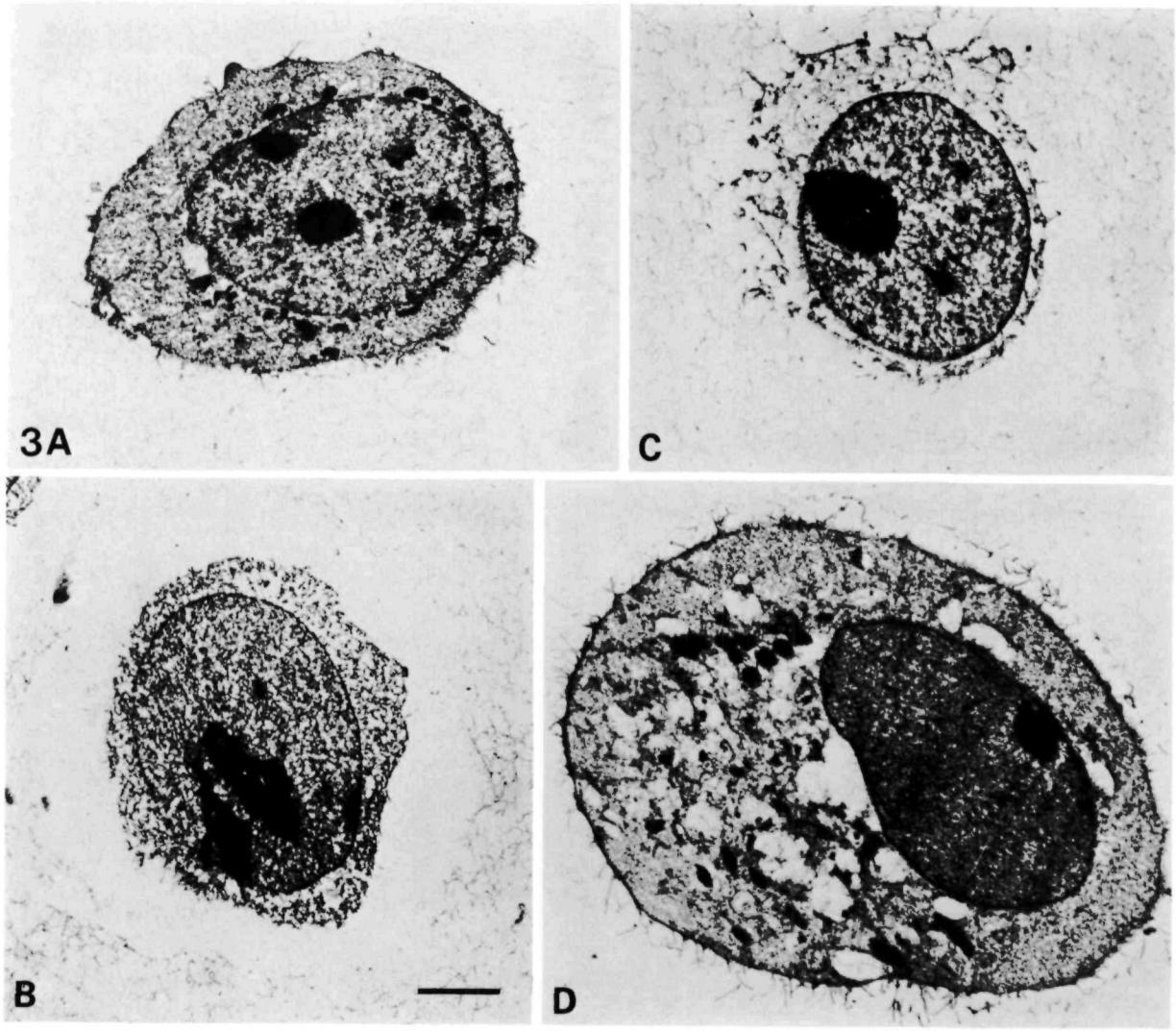

Fig. 3. Electron micrographs of thick sections of cells. A. Unlysed; B, lysed, Triton/pH 7·4; C, lysed, Triton/pH 8; D, lysed, complement/pH 7·4. All are at the same magnification. Bar, 2·5 μm.

differences in detail (D. A. Jackson & P. R. Cook, unpublished data).

Macromolecular recoveries

Table 1 and Fig. 4A illustrate the macromolecular content of cells lysed in different ways. They all contain all the DNA and nascent RNA, and variable amounts of long-lived (i.e. cytoplasmic) RNA and protein; complement-lysed cells retain the most. Triton selectively removes certain proteins, unlike complement and antibody (Fig. 4A; note that twice the number of cell equivalents were loaded in lanes 4 and 5). There was no obvious effect of pH.

When cells are heat-shocked by temperatures 3–5 deg. C above normal, some proteins associate with karyoskeletal elements. This may happen with nuclei isolated by conventional procedures, but it is then triggered by physiological temperatures; isolation of nuclei sensitizes them. The effect is especially noticeable after the heat-shocked nuclei are treated with deoxyribonuclease and extracted with 2 M-NaCl (Evan & Hancock, 1985; Littlewood *et al.* 1987; McConnell *et al.* 1987). This has led to skepticism as to whether

Table 1. *Macromolecular recoveries of encapsulated cells lysed in different ways*

	% Remaining after lysis			
		RNA		
Lysis conditions	Protein	Nascent	Long-lived	DNA
Complement, pH 7·4	67	95	62	96
Triton, pH 7·4	24	98	28	99
Complement, pH 8·0	65	94	58	101
Triton, pH 8·0	23	97	20	100

Nascent RNA and long-lived RNA were determined after labelling for 2 min or 24 h.

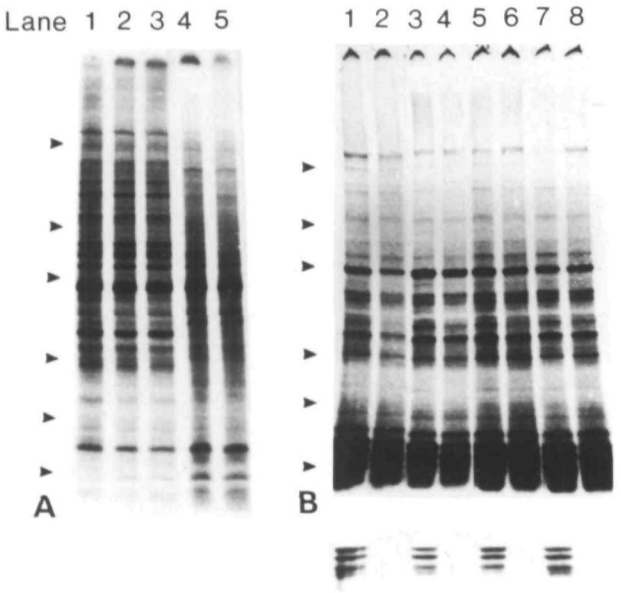

Fig. 4. Autoradiographs of gels containing proteins from cells, prelabelled with [^{35}S]methionine, and extracted in various ways (A) or extracted and heat-treated (B). Arrowheads indicate the positions of size markers of 93, 69, 46, 30, 21 and 14 ($\times 10^3$) M_r. A. Encapsulated cells, unlysed (lane 1) or lysed and washed using complement/pH 8·0 (lane 2), complement/pH 7·4 (lane 3), Triton/pH 8·0 (lane 4) and Triton/pH 7·4 (lane 5). Lanes 1–3 contain 10^5 cell equivalents and lanes 4 and 5, 2×10^5. B. Encapsulated cells, lysed in Triton/pH 7·4 were washed, residual soluble protein was removed electrophoretically under isotonic conditions and beads treated for 20 min at 0°C (lanes 1, 2), 30°C (lanes 3, 4), 37°C (lanes 5, 6) and 43°C (lanes 7, 8). After treatment, some of each sample was applied directly to the gel (odd-numbered lanes) and the remaining beads were extracted with 2 M-NaCl, 10 mM-Tris·HCl (pH 8·0), 1 mM-EDTA and washed thoroughly before applying to the gel (even-numbered lanes). Autoradiography was for 2 weeks; the inset below shows a 2-day exposure of the overexposed region at the bottom of the gel that contains the histones.

structures seen only after pretreatment of nuclei at 37°C, like scaffolds (Mirkovitch *et al.* 1984), have counterparts *in vivo* (McConnell *et al.* 1987). We therefore repeated the experiments of Littlewood *et al.* (1987) to see whether heat-shocking our preparations induced a similar set of proteins to become associated with the karyoskeleton. Cells were prelabelled with [^{35}S]methionine, encapsulated, lysed, washed, incubated at different temperatures and any residual soluble proteins were removed electrophoretically; then proteins remaining associated with beads were analysed by gel electrophoresis (Fig. 4B, odd-numbered lanes). No proteins associate with our preparations on incubation at 43°C *in vitro*, nor do they do so after extraction with 2 M-NaCl (Fig. 4B, even-numbered lanes; a shorter exposure is shown in the inset below and shows that most histones have been extracted). (Treatment with deoxyribonuclease (10 μg ml^{-1}; 30 min at 0°C or 30°C) prior to extraction with 2 M-NaCl gave similar results (results not shown).) As we shall see, functional studies also show that our preparations have not been sensitized to heat, since replication and transcription, which are shut down when living cells are heat-shocked, continue efficiently at 37°C. Perhaps nuclei prepared by conventional procedures become sensitized to heat when they are exposed to the hypotonic conditions used during cell lysis.

Permeability

Before they can reach all cell compartments, added probes must pass successively through the agarose coat and however much of the cell membrane, cytoplasm, nuclear membranes and chromatin that remains. The agarose coat offered no barrier to the probes that we used. Theory suggests 0·5% agarose is permeable to globular proteins of $<2\times10^8 M_r$ and 120-nm diameter, and practice shows that nucleoprotein complexes of $1{\cdot}5\times10^8 M_r$ pass through it (Jackson & Cook, 1985*b*). As a result, enzymes equilibrate throughout beads within seconds. For example, encapsulating DNA delays maximal transcription by added RNA polymerase from *E. coli*, one of the largest enzymes known (i.e. $4{\cdot}8\times10^5 M_r$), by less than 2 min (results not shown). However, the diffusion of larger DNA molecules is limited. Thus, the half-times for escape of λ *Hin*dIII fragments of 23·7, 9·5, 6·7, 4·3 and 2·3 kb from 0·5% beads are 200, 80, 35, 20 and 10 min, respectively (see Materials and methods). In contrast, DNA molecules the size of yeast chromosomes (>250 kb) remain trapped for at least 6 months (results not shown).

We next determined how accessible the various cellular compartments were to various probes. Although it was originally thought that Triton extracted only the cell and outer, but not inner, nuclear membrane, it is now known to remove all three (Aaronson & Blobel, 1974). Therefore, Triton-extracted encapsulated cells are accessible to a variety of molecular probes (e.g. restriction enzymes, polymerases and antibodies). Fig. 1J–M illustrates such cells stained with various antibodies (i.e. those to the nuclear pore complex on the nuclear periphery (Fig. 1J), Sm antigens inside the nucleus (Fig. 1K) and cytoplasmic vimentin fibres that have collapsed on lysis into a dense mass on the nuclear surface (Fig. 1M). An antibody directed against a protein not found in HeLa cells provides a control (Fig. 1L). Note that unlike most other immunofluorescence images, Fig. 1J and K are from unfixed cells, i.e. unfixed in the sense that many enzymic activities remain (see below).

In contrast to the permeability of most compart-

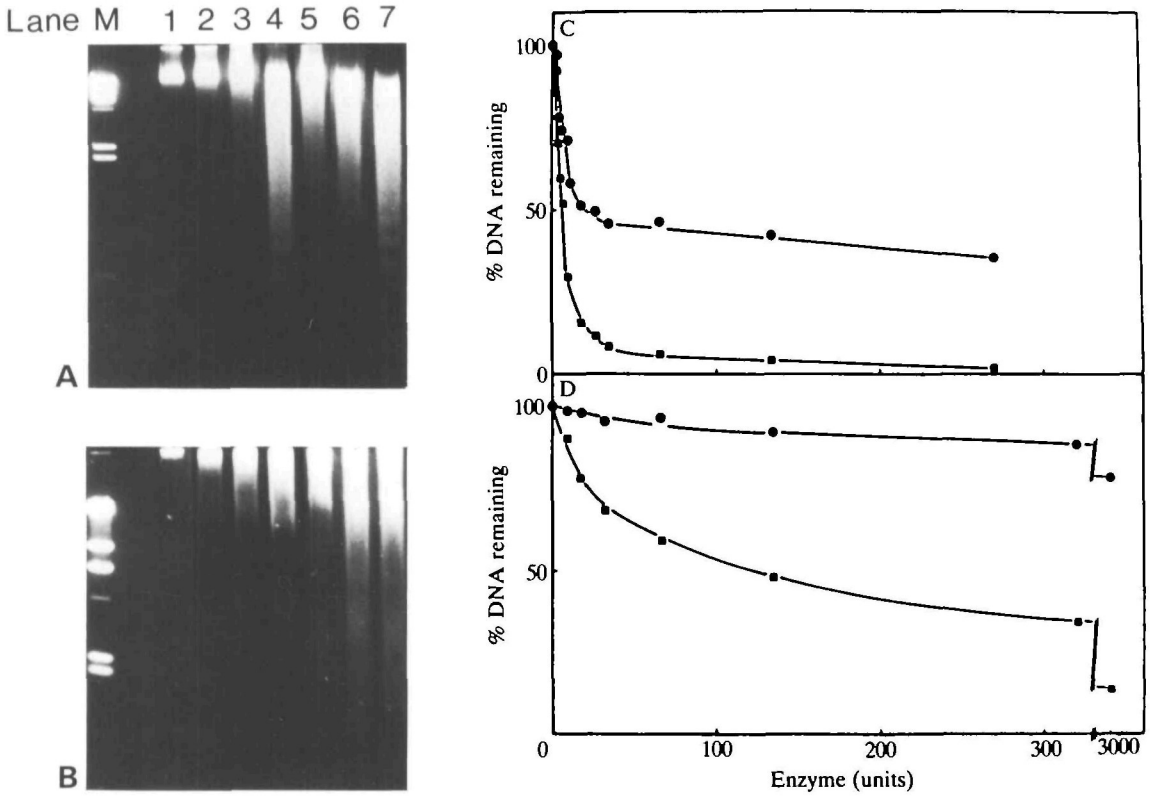

Fig. 5. Accessibility to restriction enzymes of chromatin in encapsulated cells lysed using Triton or complement at pH 7·4. Cells were labelled with [^{3}H]thymidine, encapsulated, lysed, treated with various amounts of *Hae*III (A,C) or *Eco*RI (B,D) and samples split. A,B. The size distribution of DNA was determined in one half. DNA was purified, subjected to gel electrophoresis, and the gels stained with ethidium and photographed. A. *Hae*III digestion. Lane M, marker λ DNA cut with *Hin*dIII; lane 1, uncut sample; lanes 2–4, complement-lysed cells; and lanes 5–7, Triton-lysed cells incubated with 16, 66 and 270 units, respectively. B. *Eco*RI digestion. Lane M, marker DNA; lane 1, uncut sample; lanes 2–4, complement-lysed cells; and lanes 5–7, Triton-lysed cells incubated with 42, 167 and 666 units (product of units ml^{-1} and time (h)), respectively. C,D. The percentage of chromatin that resisted electroelution from beads was determined using the other half. Beads were subjected to electrophoresis, recovered and the percentage of ^{3}H (i.e. chromatin) remaining within them determined. Digestion with *Hae*III (C) or *Eco*RI (D). (■) Triton-lysed cells; (●) complement-lysed cells.

ments of Triton-lysed cells to antibodies, the cell membrane of complement-lysed cells is impermeable (results not shown); antibodies are too bulky to pass through the 10 nm pores generated by the attack complex (Muller-Eberhard, 1986). (Note that this is true only for 'robust' cells like HeLa. The precise mechanism by which complement lyses nucleated cells remains obscure, but the membrane of some cells (e.g. lymphocytes) may tear on lysis, so that antibodies enter them (results not shown).) Of course, complement-lysed cells become permeable to antibodies after fixation; Fig. 1N illustrates an excellently preserved network of vimentin fibres. However, unfixed but complement-lysed HeLa cells are permeable to smaller molecules like rhodamine-conjugated phalloidin (Fig. 1H). The actin filaments in the cytoplasm are seen as a fine three-dimensional network, which is not reproduced well at the exposure chosen in Fig. 1H. On treatment after lysis with cytochalasin these filaments aggregated; the resulting thicker filaments fluoresced brightly (Fig. 1I; a similar exposure was used for Fig. 1H and I). Note that images of cytoskeletal networks are usually obtained using well-flattened cells attached to coverslips; here we used round cells. In addition, cytochalasin can only effect a rearrangement after lysis as the material is 'unfixed'.

We next explored the accessibility of chromatin in Triton-lysed cells to the restriction enzymes, *Hae*III and *Eco*RI (Fig. 5). (*Eco*RI is composed of two subunits of $29\times10^3 M_r$ (Modrich & Zabel, 1976); we have been unable to discover the size of *Hae*III.) Beads were incubated with the enzymes, then their DNA was subjected to electrophoresis. DNA from the zero time point is of such a high molecular weight it barely enters the gel (Fig. 5, lane 1; the slight cutting seen at 'zero' time occurred during the inevitable lag between addition of enzyme and stopping the reaction). As expected, treatment with *Hae*III cut most of the DNA in Triton-lysed cells into pieces of <20 kb, with some being cut to nucleosomal size (Fig. 5A, lane 7). *Eco*RI,

which cuts less frequently, generated larger fragments (Fig. 5B, lane 7). The enzymes also cut chromatin in complement-lysed cells, albeit to a lesser extent (Fig. 5A and B, lanes 2–4). Clearly, they pass not only through the pores generated by the attack complex but also those in the nuclear membrane. *Hin*dII ($75\times10^3 M_r$) is the largest enzyme that we have found that enters the nucleus (results not shown). (Both sets of pores are about 10 nm in diameter, slightly larger than that of 'ideal' spherical proteins the size of *Hin*dII.) If beads containing lysed cells are subjected to electrophoresis in isotonic conditions after digestion, chromatin fragments escape (Fig. 5C and D), presumably through these pores. (We suspect that electrophoresis does not tear membranes, as both nucleus and cytoplasm remain inaccessible to antibodies.) Essentially all chromatin can be electroeluted after *Hae*III treatment of Triton-lysed cells (Fig. 5C). The kinetics of removal are biphasic and probably reflect a complex interplay between an initial accessibility of hetero- and eu-chromatin and then differential electrophoretic movement of variously sized particles through residual skeletons and pores.

These results mean that the nuclear interior of both Triton- and complement-lysed preparations will probably be accessible to most enzymes currently used in molecular biology. Note also that nearly all the RNA and DNA polymerase activities of the cell are recovered in beads after cutting and electroelution (Jackson & Cook, 1985*b*, 1986*a*). (We have repeated these earlier experiments using the pH 7·4 buffer, with essentially similar results (not shown; see below).)

DNA integrity

The presence of nicks or breaks in encapsulated nuclear DNA can be detected with great sensitivity by fluorometry using the intercalating dye, ethidium bromide (Cook & Brazell, 1978; Cook, 1984). Treating lysed and encapsulated cells with 2 M-NaCl removes most proteins, leaving naked and superhelical DNA looped by attachment to a nuclear cage or matrix. One nick anywhere within a loop of about 100 kb releases all supercoiling from that loop and this loss of supercoiling increases the amount of ethidium bound to the DNA. As the fluorescence of ethidium is enhanced when it binds, binding, and hence integrity, can be conveniently monitored by fluorometry. After addition of ethidium, each sample was divided, half γ-irradiated with a dose ($9\cdot6\ J\ kg^{-1}$) sufficient to relax DNA fully and the fluorescence of both was measured. After subtraction of appropriate blanks, the fluorescence of dye bound to non-irradiated beads was divided by that bound to irradiated beads, effectively normalizing all non-irradiated values to those of equal amounts of fully relaxed DNA. Therefore, this ratio reflects the proportion of loops that are nicked. Ratios from encapsulated cells lysed directly in 2 M-NaCl and their γ-irradiated counterparts provide fully superhelical and fully relaxed DNA for comparison (with ratios of 0·86 and 1·0, respectively).

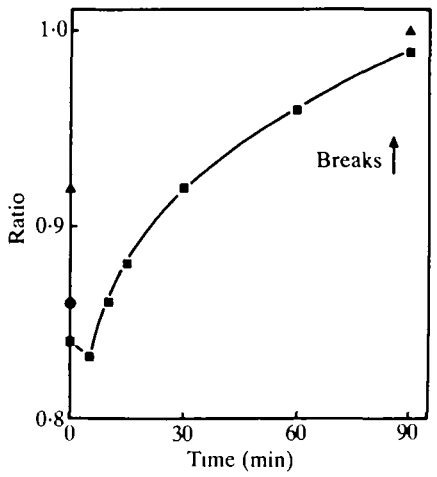

Fig. 6. Nicking DNA during incubation. Cells were encapsulated, lysed with Triton (■) or complement (▲) at pH 7·4, washed and incubated at 37°C. At different times, 2 M-NaCl was added to remove histones and generate loops of superhelical DNA. The ratio was determined fluorometrically and reflects the percentage of nicked loops, the higher the ratio, the more breaks. (●) Encapsulated cells lysed directly in Triton and 2 M-NaCl.

Most loops in encapsulated cells lysed in Triton at pH 7·4 are supercoiled, whereas some of those lysed in complement are nicked. On incubation at 37°C the DNA of both becomes progressively nicked (Fig. 6). Nicking occurs more rapidly if the Mg^{2+}/ATP ratio is increased (Jackson & Cook, 1985*a*; results not shown). Since this assay is so sensitive, this means that net nucleolytic activity is effectively suppressed and that, at least for the first few minutes, these beads contain essentially intact DNA. It also means that lysing cells with detergents or complement does not grossly damage DNA (Li & Kaminskas, 1987), as may cell-mediated cytolysis (Duke *et al.* 1983).

Efficiency of replication and transcription

Table 2 illustrates the efficiency of replication and transcription in these preparations using optimal concentrations of triphosphates. (We would stress that we assay activities due to polymerase halted at lysis, and which then continue synthesis *in vitro* without reinitiation (Jackson & Cook, 1985*b*, 1986*b*). For these experiments, extra Mg^{2+} and, of course, triphosphates were added: if maintained in equimolar amounts, nicking then occurs at roughly the rates indicated in Fig. 6.) Cells lysed in Triton replicate their chromatin template in a cell-cycle-dependent manner at >75 % of the rate *in vivo* at pH 7·75, the reaction being very sensitive to pH and Mg^{2+} concentrations (Jackson & Cook, 1986*b*). Consequently, Triton-lysed cells repli-

Table 2. *The efficiency of replication and transcription*

Lysis conditions	Replication: Rate (pmol/10^6 cells per min)	Replication: Efficiency (%)	Transcription rate (pmol/10^6 cells per min)
Complement, pH 7·4	6·4	85	9·5
Triton, pH 7·4	2·0	27	14·6
Complement, pH 8·0	9·0	120	17·5
Triton, pH 8·0	3·1	41	19·2

The efficiency of replication is determined assuming that an average nucleus contains 12 pg DNA and that one cell cycle takes 22 h (Jackson & Cook, 1986*a*).

cate slightly less efficiently at pH 7·4 and 8·0. In contrast, complement-lysed cells replicate more efficiently, replicating faster *in vitro* at pH 8·0 than *in vivo*. Nuclei prepared conventionally (without Triton) by homogenizing cells swollen in a hypotonic buffer (for example, our pH 7·4 buffer minus KCl) provide a reference level of replication for comparison; they replicate under identical conditions at only 70 % of the rate found with cells extracted with Triton at pH 7·4 (results not shown).

We explored the basis for this difference in efficiency of replication (assayed under identical conditions) of Triton- and complement-lysed cells but are unable as yet to explain it. The difference is also obtained with [^{32}P]dCTP (results not shown). It is strongly dependent on deoxynucleotide triphosphate concentration, being about threefold at optimal concentrations (i.e. 125 μM; Table 2); lower concentrations reduce the rate of Triton-lysed cells more than complement-lysed cells, so that the difference increases to about 15-fold at 2 μM (Fig. 7) and to 30-fold at lower concentrations. The difference in rate is directly attributable to Triton as addition of detergent to complement-lysed cells immediately reduces their rate (Fig. 7). This reduced rate was not due to a non-specific loss of activity on incubation, since addition of Triton at the beginning of the reaction reduced the rate to the same low level (results not shown). The activity in complement-lysed cells is sensitive to aphidicolin (Fig. 7), as are the major activities *in vivo* and in Triton-lysed cells (Jackson & Cook, 1986*a*,*b*).

Transcription is equally efficient (Table 2). As we do not know the absolute rate of transcription *in vivo* we cannot estimate relative efficiencies; however, we know of no preparation that transcribes more efficiently.

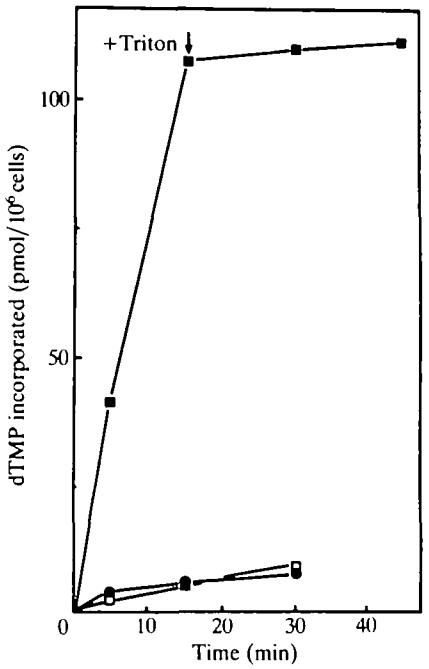

Fig. 7. The kinetics of replication in Triton- and complement-lysed cells. Replication assays were conducted as described in Materials and methods except that the dTTP concentration was reduced to 2·5 μM. (■) Complement-lysed cells. After 15 min Triton was added to 0·25 %. (□) Complement lysed cells, preincubated for 30 min with 10 g ml^{-1} aphidicolin. (●) Triton-lysed cells; preincubation with aphidicolin reduces the initial rate 15 times (results not shown) and addition of Triton to 0·25 % during the reaction has no effect.

Screening for lytic agents that selectively permeabilize the cell membrane

The observation that Triton reduces the efficiency of replication of complement-lysed cells, presumably by permeabilizing the nuclear membrane, allowed us to screen lytic agents for those that might permeabilize the cell, but not the nuclear, membrane. Radiolabelled dTTP cannot pass through the cell membrane, so addition of such a specific agent should increase incorporation of label to that of complement-lysed cells; non-specific agents should increase the level only to that of Triton-lysed cells. We tested various non-ionic detergents, the best of which, Tween 20, gave the results illustrated in Table 3; we found that many could be diluted to a concentration where most cells in the population became permeable to Trypan Blue, but this never led to replication at the rate of complement-

Table 3. *The relative efficiency of replication after lysing cells in the pH 7·4 buffer and various agents, measured using 2·5 μM-dTTP*

Lytic agent	Relative efficiency of replication
Triton X-100, 0·5 %	1·0
Tween 20, 0·5 %	2·4
Melittin, 25 $\mu g\,ml^{-1}$	1·7
Melittin 250 $\mu g\,ml^{-1}$	0·4
Complement	6·5

Values are expressed relative to cells lysed using Triton.

lysed cells; higher concentrations always reduced rates. Lysis was also variable and critically depended on cell number and type. Similar results were obtained with ionic detergents (e.g. SDS and lysolecithin; results not shown). Lysis by the protein, melittin, was also variable, high levels inhibiting replication (Table 3).

Discussion

We set out to develop a method for gently permeabilizing cells with a number of aims in mind. We hoped to use as 'physiological' a buffer as possible and to permeabilize selectively the various cellular compartments to most of the probes currently used in cell biology. We have partly achieved these aims.

Buffers

The cationic, but not anionic, constitution of our pH 7·4 buffer is roughly that of the cytoplasm. Proteins are the dominant anions *in vivo* but their inclusion in a buffer for routine use is inconvenient. However, their addition might serve a useful purpose. Although our buffer preserves heterochromatin better than most, some still decondenses when the nuclear membrane is removed (cf. Triton- and complement-lysed cells), presumably because nuclear proteins diffuse out within seconds, a loss that can be made slower by high concentrations of protein (Paine *et al.* 1983). Despite these shortcomings, we believe that our formulation provides a suitable base that can be modified as more information about the precise cellular environment becomes available.

Lytic agents

We tested various lytic agents (Table 3) to see whether any preferentially permeabilized the plasma membrane, but found none as efficient as antibody and complement. (But see, for example, Schindler *et al.* (1985), who selectively removed the outer nuclear membrane with citraconic anhydride.) Antibodies provide the advantage of specificity but the disadvantage that complement (i.e. whole serum) is ill-defined and so might have unexpected effects. For example, intermediate filaments contain high-affinity sites for the Fc part of IgG and so fix complement (Hansson *et al.* 1987). It also has the disadvantage that although its perforations (about 10 nm) permit ingress of restriction enzymes they are too small for antibodies. (Note that the membrane of cells with less well-developed cytoskeletons than HeLa may tear on complement-mediated lysis so that their interiors do become accessible.) We are currently exploring whether the nuclear membrane of complement-lysed cells is functionally intact. We also permeabilized the plasma membrane using strong electric fields, but found that this is best achieved in buffers of low ionic strength, which reduce the rates of transcription and replication (Knight & Scrutton, 1986). (Note that by virtue of its larger radius, the cell membrane becomes permeable at lower fields than those of intracellular organelles (Knight & Scrutton, 1986).)

Preservation of structure

The agarose coat protects the fragile contents of cells very effectively. For example, the three-dimensional structure of the cytoskeleton survives repeated pelleting and resuspension, as does the fragile DNA. The coat also provides the added benefit of packaging viscous DNA, which, if released, makes handling difficult. Most of the experiments that we describe would be impossible using nuclei prepared using conventional procedures, since they aggregate and jellify at physiological salt concentrations.

Preservation of function

A major concern in our laboratory has been with nuclear function, replication and transcription. We have shown elsewhere that after lysis in Triton at pH 8·0 the encapsulated cells replicate their DNA *in vitro* at 85 % of the rate found *in vivo* in a cell-cycle-dependent manner (Jackson & Cook, 1986*b*) and they transcribe more efficiently than nuclei prepared conventionally (Jackson & Cook, 1985*b*). The rates on lysis with Triton in our improved buffer are similarly high. Surprisingly, complement-lysed cells replicate under identical conditions about three times more efficiently than their Triton-lysed counterparts (Table 2) and it is perhaps premature to speculate on the basis of this interesting phenomenon. However, it is interesting that efficient replication in extracts of *Xenopus* oocytes is preceded by the re-formation of an intact nuclear membrane (Blow & Laskey, 1986; Blow & Watson, 1987; Newport, 1987), so that membrane integrity may be a prerequisite for efficient replication.

We have not explored cytoplasmic function in any detail, but we would assume that as complement-lysis preserves cytoplasmic morphology quite well, many functions in addition to the cytochalasin-induced col-

lapse of the actin network (Fig. 1I) may also be preserved.

Accessibility

The agarose pores are so large in comparison with the size of the average protein that antibodies and enzymes equilibrate throughout beads within seconds. After lysis with Triton, both cytoplasm and nucleus become freely accessible to antibodies and, to a lesser extent, to larger nucleic acids (results not shown). After complement-mediated lysis, the pores generated by the attack complex (Muller-Eberhard, 1986) do not permit ingress of large proteins like antibodies unless the membrane is torn, but their chromatin is accessible to restriction enzymes and so probably to most enzymes used in molecular biology.

We hope that these encapsulated derivatives of cells, well-preserved and containing intact DNA, cytoskeleton and nucleoskeleton, accessible, yet expressing *in vitro* the major nuclear functions at rates found *in vivo*, provide a useful experimental material for studies on the relations between the various structures in the cell and their functions.

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