The nucleus of axenically grown *Dictyostelium discoideum:* studies on its division cycle, isolation and conformation

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Summary. The saponin digitonin is proposed as an alternative lysing agent to non-ionic detergents, for the preparation of isolated nuclei from axenic cultures of *Dictyostelium discoideum.* Digitonin-isolated nuclei are compared with those isolated using conventional detergents by means of light and scanning electron microscopy, transcriptional activity and the extent of DNA degradation. The appearance of multinucleate cells in axenic cultures and certain other aspects of axenic growth are also discussed.
Key words. Dictyostelium; axenic culture; digitonin; isolated nuclei.

Introduction

The cellular slime mold *Dictyostelium discoideum* exists during the dispersed phase of its life-cycle as separate, unicellular, motile, ameboid cells. These myxamoebae are commonly grown in association with a variety of species of bacteria on agar plates; however, certain strains can be grown axenically as suspended cells in a shaken liquid culture vessel[30, 31]. Such cultures provide a rapidly proliferating, homogenous population of easily cultured cells well suited to molecular and biochemical analysis.

The two most commonly use axenic strains, both derived from the nonaxenic strain Nc-4, are Ax-2[1, 31] and Ax-3[4, 5, 8, 28].

A number of laboratories have been involved in the isolation of nuclei from this organism and a variety of preparative techniques have been used, most relying on the use of detergents such as NP-40[2], Triton X100[4, 5] and Cemulsol NPT12[5], although some other surface active compounds such as amphotericin B (Fungizone)[18] have also been tried.

Using the saponin (saponins are plant-derived compounds with detergent properties), digitonin, we have sought to produce an improved method for the preparation of isolated nuclei and have gone on to study the characteristics of the nuclei so obtained. In this paper we describe such a method for nuclear isolation using the axenic strain of *D. discoideum* Ax-2. Comparative data is presented on characteristics of such nuclei as compared to nuclei prepared using more conventional methods. The nuclei were compared in terms of the effect of isolation on their nuclear envelopes, transcriptional activity and chromatin conformation.

Materials and methods

Growth condition

Dictyostelium discoideum, strain Ax-2 was grown in 700-ml volumes in 2-l conical flasks at 21 °C on a rotary shaker in the following axenic medium: oxoid bacteriological peptone, 14.3 g; oxoid yeast extract, 7.15 g (both from oxoid U.K. Ltd.); D-glucose, 15.4 g; Na_2HPO_4 12 H_2O, 1.28 g; KH_2PO_4, 0.48 g; pH 6.7, distilled water added to make up to 1 l[31]. Log phase cells were harvested at between 1 and 2 × 10^6 cells/ml. Cells were washed once in distilled water by centrifugation at 500 × g for 5 min before lysis.

Analysis of variation in numbers of nuclei/cell with cell density, in axenic cultures

Axenic cultures were set up as described above and increases in cell numbers determined by hemocytometer counts. A series of cell densities were assayed within the range used, i.e. the linear phase of the growth curve, between about 2 × 10^5 and 6 × 10^6 cells/ml. In addition, cells were also assayed when entering stationary phase (around 10^7 cells/ml).

At a given cell density, washed cells were resuspended in lysis buffer (see 'Isolation of nuclei' below) at a concentration of 1 × 10^8/ml and 0.1 mg/ml digitonin added. Cells were stirred at room temperature with a magnetic stirrer until the majority of cells had lost their refractility but few cells had actually lysed.

Numbers of nuclei per cell were determined by using a × 50 or × 90 oil immersion objective.

Counts of no less than 300 cells, in non-overlapping fields of view, were taken at each cell concentration (this being determined as the minimum number to give statistically significant average values for numbers of nuclei/cell).

Fluorescence microscopy

Slides were prepared with 1 drop of cell suspension added to 1 drop of acridine orange (0.01% in Sorensens Buffer) and viewed under a Leitz Ortholux Fluorescence Microscope with exciting light at 470 nm (blue).

Isolation of nuclei

Nuclei were isolated by a modification of the procedure of Jacobson[16] and Charlesworth and Parish[4]. Washed cells were resuspended in lysis buffer; 50 mM hepes (pH 7.5); 5 mM $MgAc_2$; 10% sucrose; 40 mM KCl; 1 mM (phenylmethylsulfonyl fluoride) PMSF at 5 × 10^7 cells/ml for lysis with 0.1 mg/ml digitonin or 10^8 cells/ml for lysis with 0.1% NP-40.

The suspension was stirred at room temperature on a magnetic stirrer until cell lysis, monitored under phase contrast microscopy, was complete: with digitonin this took 15–20 min and with Nonidet P-40 5 min. With the digitonin-lysed cells 10 strokes of a homogenizer were required to free nuclei from cellular material. All subsequent operations were carried out at 4 °C. The cell

lysate was transferred to 30 ml centrifuge tubes and nuclei collected by centrifuging at 2000 × g for 5 min in a Sorval H-400 rotor. The resulting pellet was resuspended in lysis buffer minus detergent and centrifuged again at 2000 × g. This was repeated two or three times. Any remaining whole cells in the pellet were removed by centrifuging at 700 × g for 2 min. The supernatant was then layered over 10 ml 1 M sucrose in lysis buffer and centrifuged at 5000 × g for 10 min. The nuclear pellets were resuspended in 5 ml lysis buffer and underlayed with 10 ml 1 M sucrose as above and centrifuged again.

Preparation of nuclei for electron microscopy

Nuclei in lysis buffer at approx. 10^8/ml were pipetted as a 0.05 ml aliquot onto a 1-µm nucleopore filter placed in the cap of an embedding capsule. Nuclei were fixed in 5% glutaraldehyde in lysis buffer for 30 min, then washed three times in lysis buffer, post fixed in 1% osmium tetroxide for 1 h, washed three times as before, dehydrated through a series of acetone dilutions (5 min in each), 30%, 50%, 70%, 90%, 100% and the capsules left in 100% acetone overnight. The preparations were then critical-point dried from acetone in a Poloron E2000 critical point dryer, filters coated with Au/Pd in a SEM Prep sputter coater and nuclei examined with a JEOL JJM PIS scanning electron microscope. Photographs were taken on Ilford FP4.

In vitro transcription of isolated nuclei

Nuclei were purified as described above. When retained in storage they were kept in storage buffer (40 mM Tris-HCl [pH 7.9]; 10 mM $MgCl_2$; 0.1 mM Na_2EDTA; 1 mM Dithiothreitol; 50% glycerol). Normally, however, nuclei were prepared freshly for each experiment. The reaction was initiated by the addition of 10^7 nuclei (0.05 ml) to a total reaction volume of 0.2 ml containing 40 mM Tris-HCl (pH 7.9); 10 mM $MgCl_2$; 20 mM KCl; 0.1 mM DTT; 5% glycerol; 0.6 mM ATP; 0.6 mM CTP; 0.6 mM GTP and 50 µCi ^{3}H-UTP (2 Ci/mmole, Amersham). α-amanitin and actinomycin D used at concentrations shown in figure 9. 10-µl samples were taken at the times indicated in the text and pipetted onto Whatman DE81 discs. The discs were washed 5 times in 0.5 M Na_2HPO_4, twice in H_2O, twice in ethanol, once in ether, dried and counted for 10 min in a Beckman scintillation counter.

DNA preparation and electrophoresis

Nuclei which had been prepared as described above were lysed by adding an equal volume of 100 mM Tris HCl (pH 8.0); 20 mM EDTA; 0.4 mM NaCl; 1% SDS. Proteinase K was added to 0.2 mg/ml and the sample heated at 37°C for 2 h.

DNA was then extracted by adding an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), then extracted twice with two volumes of cloroform:isoamyl alcohol (24:1) and the DNA precipitated with 2 volumes of ethanol at −20°C. The DNA was then resuspended in TE buffer, 10 mM Tris HCl (pH 8.0), 1 mM EDTA. DNA samples were electrophoresed on a 0.8% agarose gel in TEB (89 mM Tris HCl [pH 8.3]; 2.5 mM EDTA; 89 mM boric acid) at 100 V for 3–4 h and later stained with ethidium bromide.

Materials

All reagents used were of analytical grade from British Drug Houses, Poole, Dorset, England. Digitonin, proteinase-K, ethidium bromide, and nucleotide triphosphates were obtained from Sigma (London) Chemical Co. Ltd. Agarose (electrophoresis grade) from Bethesda Research Laboratories, Cambridge, England, *E. coli* RNA polymerase obtained from Miles Laboratories.

Results

Cell growth in axenic culture

Axenic cultures provide a useful source of large numbers of nearly uniform cells.

The growth curve for *D. discoideum* Ax-2, grown under the culture conditions decribed, has the following characteristics. After an initial lag period the cells show exponential growth with a doubling time of 8 to 10 h, until the cell density reaches about 6×10^6 cells/ml when the cells start to enter stationary phase, finally reached at about 2×10^7/ml. These observations are similar to those made by other authors[31].

In such exponentially growing axenic cultures, multinucleate cells may be observed[11-13,32] (fig. 1 and 2). Generally cells with one, two, three or four nuclei are seen but in our cultures under varying conditions we have observed up to seven nuclei: Zada-Hames and Ashworth[32] quote up to 20 in one cell. In agreement with Zada-Hames and Ashworth we have found the proportion of cells that are multinucleate to be variable.

In cultures of Ax-2 grown on bacterial lawns on agar (and indeed of nonaxenic strains such as NC-4[32]) the

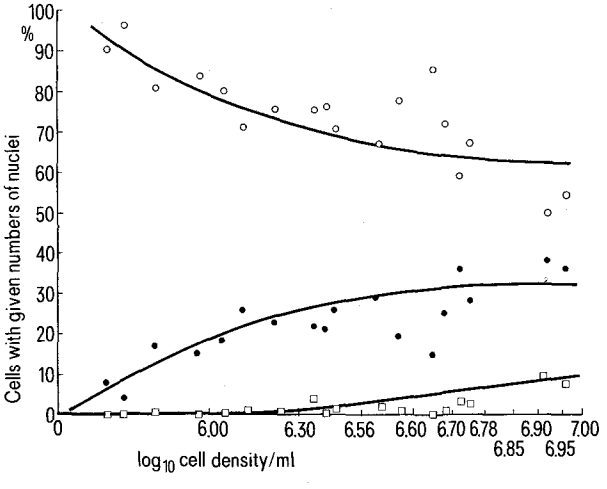

Figure 1. Analysis of variation in numbers of nuclei/cell with cell density in axenic cultures of *Dictyostelium* (see materials and methods). —○— uni-nucleate cells, —●— bi-nucleate cells, —□— cells with greater than two nuclei. (In general these are tri- and tetra-nucleates although at high cell densities cells with more than four nuclei have been observed.)

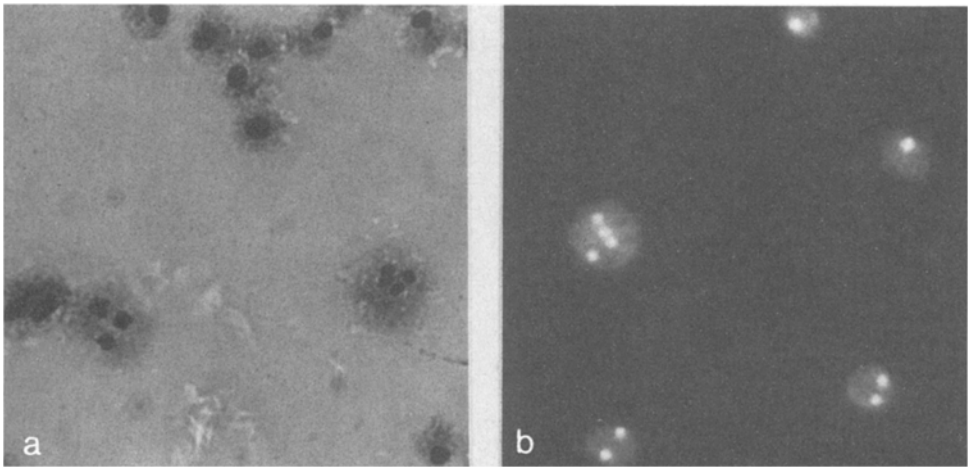

Figure 2. Photographs of multinucleate cells recovered from axenic cultures of *Dictyostelium*. *a* Cells fixed and stained according to the methods of Brody and Williams[3]. × 1200. *b* Cells photographed under the fluorescence microscope after pretreatment as described in materials and methods. × 1575.

situation is different, more than 80% of the cells being mononucleate, the remainder binucleate, and no cells with more than two nuclei.

The *Dictyostelium* nucleus possesses conspicuous nucleoli (positive identification as such comes from fluorescence microscopy of nuclear preparations stained with acridine orange, where the nucleoli, due to their associated RNA, fluoresce a characteristic bright orange against the green of the rest of the nucleus). It has been suggested that the appearance of multiple nucleoli within a single nucleus is illusary in that such nucleoli are essentially lobes of a single connected structure[22]. In our preparations nuclei can be clearly seen (by altering the focal plane during phase microscopy) to have two or four morphologically discrete, rounded nucleoli closely associated with the nuclear membrane, and so our results do not support the above interpretation.

Nuclear isolation

The use of digitonin, in preference to other detergents used to lyse *Dictyostelium* cells, provided us with clean, dark, spherical and apparently undamaged nuclei under phase contrast microscopy, and of a size which compares well with the nuclear dimensions within the living cell (fig. 3a). This compares with the NP-40 prepared nuclei which are nonspherical, with nuclear membranes often apparently collapsed or contracted around the very prominent nucleoli (fig. 3b). Similarly we have experimented with the detergent Triton X-100 and find its action very similar to that of NP-40. The NP-40 prepared nuclei in figure 5 represent an unusually good preparation and normally NP-40 prepared nuclei, both in our own and other laboratories, are as illustrated in figure 3b.

The nuclear yields obtained with digitonin depend on the required purity of the final preparation; for a very pure sample yields of 30–40% were obtained, but with cruder nuclear samples this value could be doubled. Using NP-40 a consistant yield of about 60% clean nuclei was normally obtained.

Various buffer systems were tried, including those previously employed by Jacobson[16], Bakke and Bonner[2], and Pederson[25].

Gel electrophoresis of DNA

With all variations in buffer-system, endogenous nuclease activity proved to be a problem. This was monitored by extraction of DNA from nuclear samples and running of this DNA on agarose gels (see materials and methods). In agreement with Pederson[25] we found that increasing EDTA concentrations hindered cell lysis

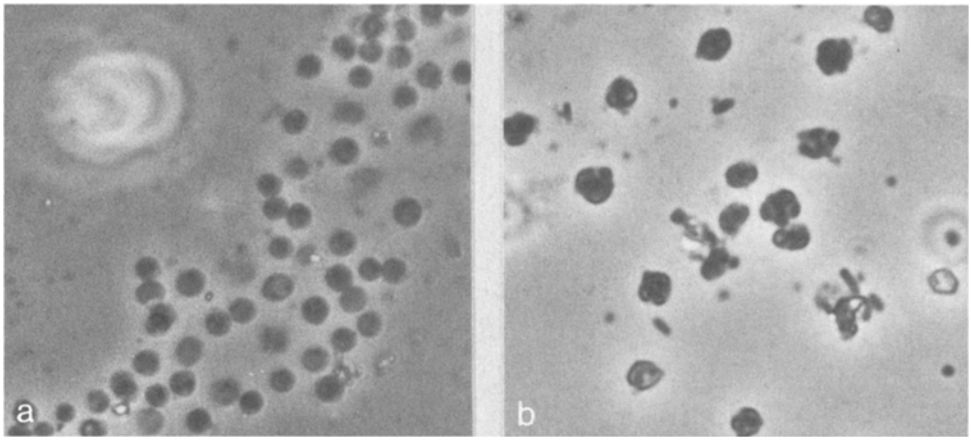

Figure 3. Light microscope photographs of preparations of isolated nuclei. *a* prepared with Digitonin at 0.2 mg/ml as described. × 2000. *b* Light microscope photograph of isolated nuclei prepared with 0.1% NP-40 as described. Note the prominent nucleoli as compared to those in figure 4a. × 2200.

resulting in longer exposure to endogenous nucleases and subsequent cutting of the DNA (fig. 4, lanes 2–5). It is possible to combat this nuclease activity in NP-40 lysis by lowering the temperature to 4 °C (fig. 4, lane 8). Digitonin lysis requires an elevated temperature, so homogenization is employed to reduce the time nuclei spend in lysis medium. In addition, increasing magnesium ion concentration helps to preserve the chromatin configuration as does the presence of KCl (fig. 4, lanes 9–11). 1 mM PMSF was included in the lysis buffer to inhibit protease activity.

Microscopy of isolated nuclei

Certain aspects of nuclear morphology became evident during the application of the isolation techniques described. One of the most noticeable was the appearance of 'doublets' in the nuclear preparations. These are seen as two daughter nuclei of a nuclear division which, although almost separated, are still enclosed in an outer membrane. It appears that these represent a stage in nuclear division when the inner membranes of the nuclear envelope become involved in the partition of the two daughter nuclei whilst the outer membrane does not (fig. 5). Such nuclei were seen at low frequencies after lysis of cells with digitonin and NP-40, although at lower frequencies with the latter. It is of course possible that this is due to a lifting off of the outer nuclear membrane due to the action of the lysing agent but the occurrence in digitonin preparations make this seem less likely (because of the selective action of digitonin on the plasma membrane).

Scanning electron microscopy of isolated *Dictyostelium* nuclei prepared from cells lysed both with digitonin and NP-40 was undertaken. These nuclei are shown in figure 6. The main difference between the two preparations lies in the appearance of the surface membrane of the nuclei, those isolated with digitonin having a relatively regular surface with few blebs, while the NP-40 nuclei have a more puckered surface to the nuclear envelope. Some of the surface irregularity in the NP-40 nuclei is presumably due to the protrusion of the nucleolar material revealed in the light microscope picture 3.b.

Transcription in isolated nuclei

Figure 7 illustrates the kinetics of (^{3}H)-UTP incorporation into RNA in nuclei isolated from cells lysed with digitonin and NP-40. Incorporation is almost linear over the first 10 min, with a decrease in linearity over the next 20 min. Incorporation continues actively for up to 120 min (results not shown). These results are in agreement with Soll and Sussman[29]. Incorporation into NP-40 nuclei with endogenous polymerase follows a similar pattern to that of digitonin nuclei, but at a considerably reduced level. The addition of *E. coli* RNA polymerase to the incubation gives a reverse result in that there is a higher incorporation into NP-40 nuclei than digitonin nuclei, 29% more at 30-min-incubation. Another difference in both cases is that with *E. coli* RNA polymerase, the incorporation is nearly linear for the 30-min-period shown; even after 120 min there is little drop in the rate of incorporation (results not

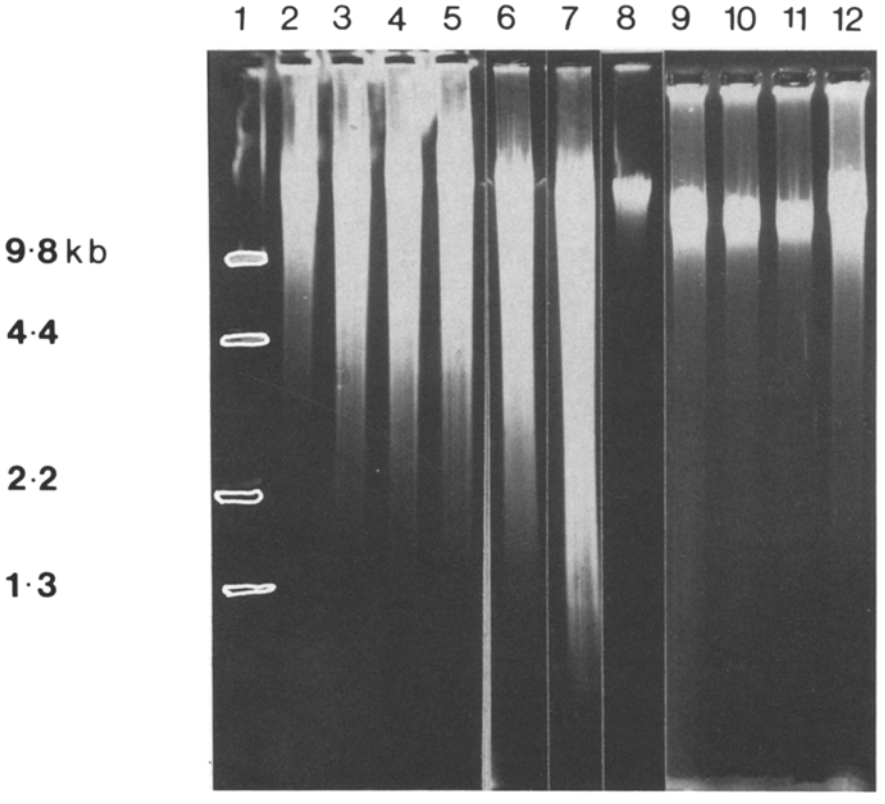

Figure 4. DNA extracted from isolated nuclei as described in materials and methods, run on a 0.8% agarose gel, and stained with ethidium bromide. Lane 1: BAM H1 Digest of *Xenopus* globin genomic DNA $\alpha\beta$1–2. Lanes 2–5: Nuclei isolated in increasing EDTA concentration 0, 0.1, 0.1, 1 mM respectively; cells lyses with digitonin. Lanes 6–7: Cells lysed in 1 mM $MgAc_2$ (no KCl) with digitonin and NP-40 respectively at room temperature. Lane 8: Cells lysed with NP-40 at 4 °C, nuclei spun out of lysate as soon as possible. Lanes 9–11: Cells lysed with digitonin at room temperature in 3 mM $MgAc_2$, 3 mM $MgAc_2$ + 40 mM KCl, and 5 mM $MgAc_2$ + 40 mM KCl respectively. Cells spun out of lysate as soon as possible as in procedure now adopted. Lane 12: Cells lysed with digitonin at room temperature in 5 mM $MgAc_2$ (no KCl).

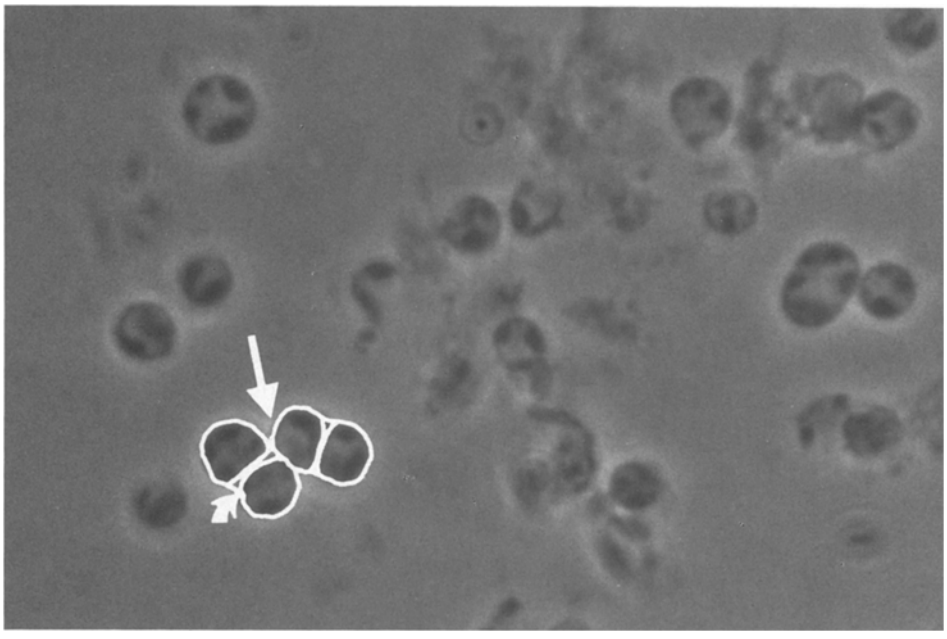

Figure 5. Light microscope photographs of isolated nuclear doublets as described in text, isolated with NP-40. This was a particularly good nuclear preparation with NP-40. × 3520. Large arrow indicates two such doublets just prior to completion of division. Small arrows indicate the still intact outer membrane.

shown). Plasmid DNA, present in the same concentration as that of total DNA in the nuclei used, was employed as a control to monitor nuclease activity and response to *E. coli* polymerase. The results shown in figure 7 are from one experiment but are typical of a number of different experiments. Data from other such experiments is given in table 1, where it will be noted that although incorporation rates vary between experiments, the relative differences in the level of incorporation in the different samples remain.

As seen in figure 8, there was a decrease of about 65% of the (^{3}H)-UTP incorporated into RNA during the first 15 min in the presence of α-amanitin; little difference was apparent in using 2 or 200 μg/ml α-amanitin. In the presence of 2 μg/ml actinomycin D there was a reduction of 83% (^{3}H)-UTP incorporated into RNA and with 200 μg/ml actinomycin D of 92%. The use of α-amanitin and actinomycin D together only permits about 1–2% activity of the endogenous polymerase after 15 min. As for figure 7, the results in figure 8 are from one experiment but typical of a series of experiments.

Discussion

Axenic culture

All the experiments described have utilized nuclei isolated from axenic cultures of *Dictyostelium*. As stated in the introduction, such cultures have various advantages over bacterially grown cells; the prime ones being provision of an easily cultured supply of large numbers of cells and eliminating the problem of removal of bacteria from harvested amoebae.

In general, it has been assumed that axenic strains do not differ significantly from bacterially grown ones. Little work has been done to assess any differences which may exist. Some ultrastructural analysis[22] has been carried out. At this level, the only apparent differences lie in the size of the osmoregulatory apparatus

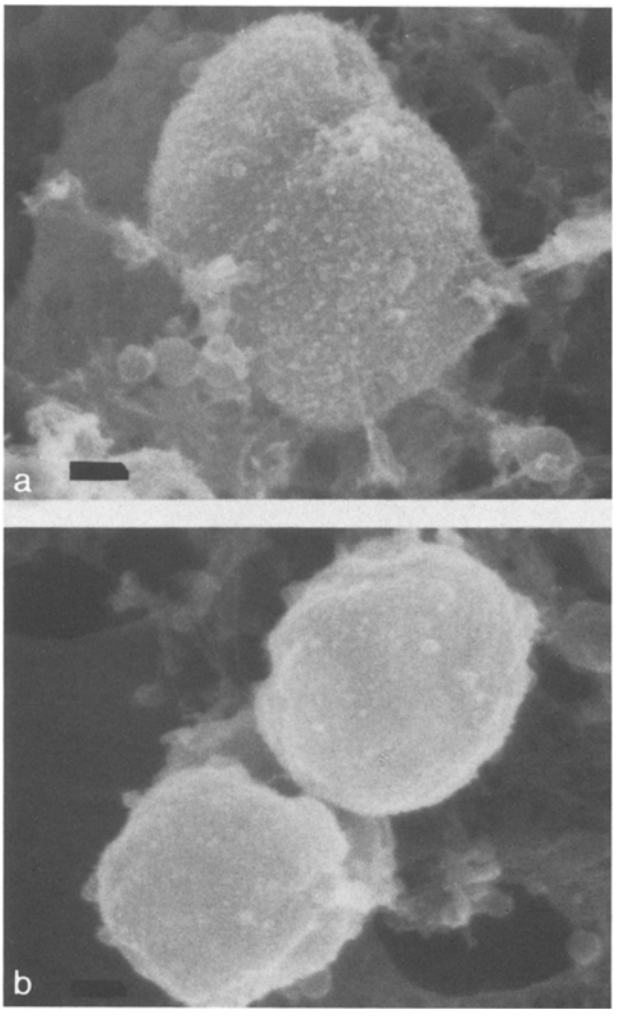

Figure 6. Scanning electron microscopy of isolated nuclei. *a* Scanning electron microscope photograph of an isolated nucleus prepared with digitonin. × 37,500. *b* Scanning electron microscope photograph of an isolated nucleus prepared with 0.1% NP-40. × 37,500.

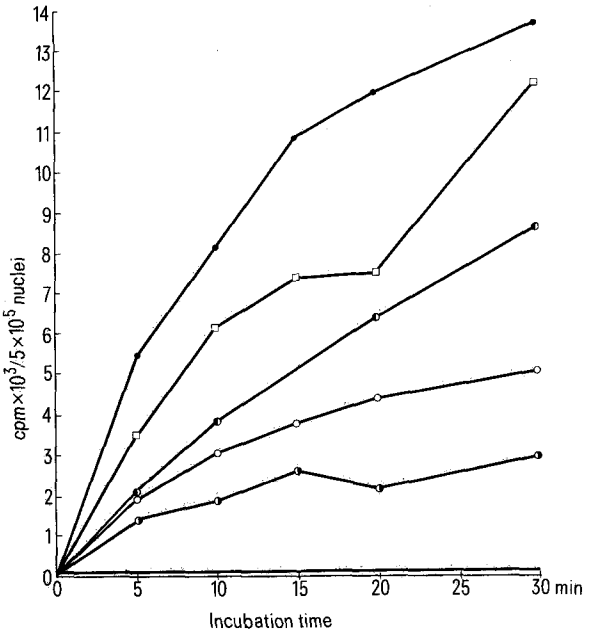

Figure 7. Time course of incorporation of tritiated uridine into RNA in isolated nuclei. Nuclei were isolated as described in materials and methods. 10 µl aliquots removed at designated intervals, and incorporation plotted as a function of time at 22 °C. —●—, pBR 322 DNA plus *E. coli* RNA polymerase; —□—, NP-40 nuclei plus *E. coli* RNA polymerase; —◐— digitonin nuclei plus *E. coli* RNA polymerase; —○—, digitonin nuclei using endogenous polymerases; —◑—, NP-40 nuclei using endogenous polymerases; — control with no pBR 322 DNA. Conditions of incubation and provision of label as described in materials and methods.

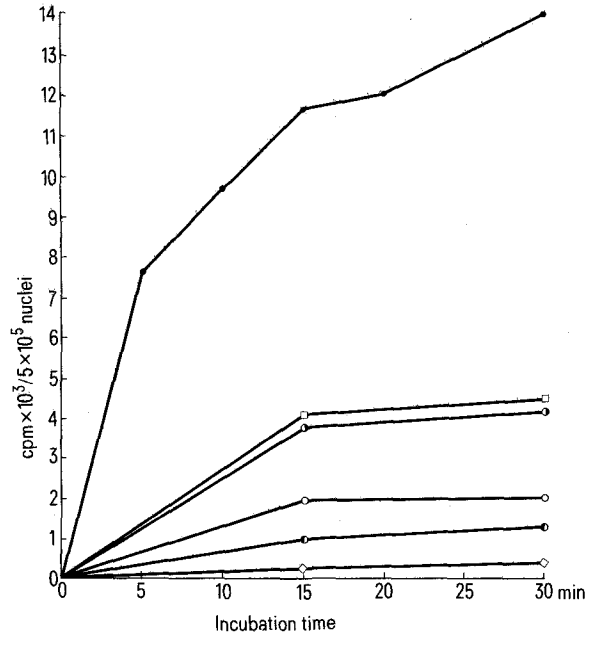

Figure 8. Time course of incorporation of tritiated uridine into RNA in digitonin isolated nuclei in the presence or absence of α-amanitin and actinomycin D. Conditions for synthesis identical to those described for figure 7. —●—, endogenous RNA polymerase activity; —□—, 2 µg/ml α-amanitin; —◑—, 200 µg/ml α-amanitin; —◐—, 2 µg/ml actinomycin D; —○—, 200 µg/ml actinomycin D; —◇—, 50 µg/ml α-amanitin + 50 µg/ml actinomycin D.

(which is, not surprisingly, larger in axenically grown cells), and in the presence of multilaminar membrane whorls in bacterially grown cells only, presumed to be related to this feeding mode. The 'abnormal' environment with its loss of substrate contact almost certainly affects the cell in other ways.

One example of the differences between bacterially grown and axenically grown cells is the presence of multinucleates in the latter. The absence of this in cells grown on bacteria suggests that some feature of axenic growth is responsible. There is some genetic evidence that a very small proportion ($< 10^{-4}$) of multinucleates must arise from cell and nuclear fusion[17] and on very rare occasions such have been seen under the microscope[13,14]. Such a phenomenon, can, however only account for a low percentage of multinucleates seen. Zada-Hames and Ashworth[32] suggest that failed cytokinesis is responsible and their cytological and cell cycle evidence goes some way to support this.

The nucleolus and ploidy in axenic cultures

It is generally assumed that *D. discoideum* myxamoebae are haploid; in all strains from the original isolate NC-4, where chromosomes have been visualized, seven have been seen and this uneven number is taken to indicate the haploid state[3,22,24]. The exceptions of known diploid amoebae are in specific 'diploid' strains, e.g. NC-4, V-12[27].

Our observation of the presence of two of four distinct nucleoli in the nuclei from cultures of Ax-2 therefore suggests that the haploid number of nucleoli is two and that four nucleoli, when seen, represent a dividing haploid cell after replication of the multiple extra-chromosomal rRNA sequences but prior to nuclear cell division.

Use of digitonin

Digitonin is a saponin and a 'sterol-specific' complexing agent, acting by binding to sterol molecules such as ergosterol and cholesterol in the plasma membrane of cells. This binding action causes permeability changes in the membrane, loss of its role as a 'selective restraining barrier' and leads to loss of cytoplasmic materials, a mode of action identical to that of the polyene antibodies such as filipin[19]. This property of digitonin and filipin renders them very toxic to fungi but not to bacteria, which lack sterols in their cell membranes. Another use of both digitonin and filipin, due to their sterol-binding action, has been in the preparation of purified plasma membrane[26].

Because of its sterol-specific activity there are sound reasons for the choice of digitonin as a lytic agent in the production of isolated nuclei. Cells treated with digitonin and filipin have been subjected to thinsection and freeze-fracture electron microscopy[6,23] and such studies have detected sterol-complexes in the plasma membranes, phagosome, digestive vacuole and autophagic vacuole membranes. They are not found in the endoplasmic reticulum, mitochondrial membranes or nuclear membranes. Treatment of cells with digitonin would

thus be expected to cause damage to the plasma membrane allowing easy disruption by homogenization but without damage to the nuclear membrane. Nonspecific lysing agents such as the detergents Cemusol[5,8], Sarcosyl[9] or Nonidet P-40[2] all of which have been used for *Dictyostelium* nuclear preparation, have the disadvantage that they cause nonspecific membrane damage by solubilization of the phospholipid components.

One disadvantage with digitonin is the requirement for an elevated temperature and longer time required for lysis, which may activate enzymes such as nucleases, proteinases and phospholipases[7,15]. We have attempted to prevent such enzymic degradation by careful design of our suspension medium.

Taking into account such necessary measures to limit enzymic activity, our evidence suggests that the use of digitonin provides isolated nuclei closely similar to those in the intact cell.

Ingredients of isolation medium

We have experimented with a wide variety of different media and our eventual choice of the lysis buffer described, 5 mM magnesium, is lower than that likely to be physiological within the cell nucleus but gives optimal results. Similarly 40 mM KCl is better in terms of nuclear survival and appearance than a higher or lower molarity.

Transcriptional activity

Endogenous synthesis of RNA in the presence of a radioactive precursor demands the availability of suitable template and the presence of active RNA polymerase enzymes. In this regard the digitonin-derived nuclei have a higher response than do the NP-40 nuclei. In the presence of added *E. coli* polymerase, the relative activities are reversed, a situation which we suggest reflects the greater availability of free or available DNA in the NP-40 nuclei due to histone depletion and other disturbance of the native conformational state of the chromatin. The responses of digitonin-prepared nuclei to added actinomycin D and α-amanitin suggest that the proportions of RNA polymerase I and RNA polymerase II-dependent transcription are approximately 83% and 65% of the total RNA synthetic activity, respectively. This clearly implies that the normal specificity of these drugs for an individual RNA polymerase, when used at an appropriate concentration, does not apply in *Dictyostelium*. The response of lower eukaryotes to these inhibitory drugs is known to deviate from the familiar pattern in higher cells[20]. In higher eukaryotes α-amanitin at 2 μg/ml gives complete suppression of polymerase II but no effect on polymerases I and III; actinomycin D at 2 μg/ml gives almost complete suppression of polymerase I, with little effect on polymerases II and III[21].

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Biological effects of trilostane in vitro on oocyte maturation and fertilization in the hamster

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Summary. The effects of the inhibition of steroidogenesis by trilostane on oocyte maturation were examined by studying spontaneous maturation and fertilization in vitro. 10^{-6} M trilostane had no influence on the meiotic process, whether the oocytes were naked or not. At a concentration of 10^{-6} M and 10^{-7} M trilostane, low normal pronuclear formation and high polyspermy were found during in vitro fertilization. However, no retarded male pronuclear development could be detected in the trilostane-treated group. Thus, steroid producing activity within ova is apparently necessary to prevent multiple sperm penetration, but it has no effect on meiosis or the action of the so-called male pronucleus growth factor (MPGF).
Key words. Hamster oocytes; oocytes, hamster; trilostane; oocyte fertilization in vitro; oocyte maturation in vitro; steroidogenesis; pronuclear formation; polyspermy.

Introduction

There has been no definite determination of the various roles of steroids in mammalian oocyte maturation and subsequent fertilization. In particular, the significance of steroids in regulating the resumption of meiosis and nuclear maturation of oocytes is still controversia[1,5,10,11,20] with regard to the cytoplasmic maturation of oocytes, some reports have appeared on the importance of steroids for the completion of maturation and the synthesis of male pronucleus growth factor (MPGF)[12,18]. Steroid-producing activity in hamster[7] and human follicular oocytes[13] has been studied by cytochemical and indirect immunofluorescence and found to be that of Δ^5-3β-hydroxysteroid dehydrogenase (Δ^5-3β-HSD). Adenylate cyclase activity has also been detected in hamster[7] and human follicular oocytes[13] by ultrastructural-cytochemical analysis. Thus, on the basis of these findings, steroidogenesis and the adenylate cyclase-cyclic adenosine 3′:5′-monophosphate (cAMP) system seem to be related. In our previous paper, the dose-dependent inhibition of Δ^5-3β-HSD activity by 10^{-6} to 10^{-8} M trilostane (Mochida Pharmaceutical Company, Tokyo, Japan) in hamster ova was reported[14]. In this study, we investigated the effects on meiotic maturation and fertilization in vitro of blocking the key enzyme, Δ^5-3β-HSD, inside ova by trilostane, in order to assess full maturation in the hamster.

Materials and methods

Female golden hamsters 9–12 weeks old with a normal reproductive cycle were used for the in vitro maturation experiments, and 6–10-week-old females and 8–12-week-old males were used for in vitro fertilization.

In vitro maturation of oocytes from preovulatory follicles. The females were injected i.p. with 30 i.u. pregnant mare serum gonadotropin (PMSG, Teikoku Zoki, Tokyo, Japan) and the ovaries were removed 48 h later[4]. Cumulus-oocyte complexes were recovered by puncturing the antral follicles with a 26-gauge needle in a watch glass filled with equilibrated medium. The complexes were washed and incubated in 20 µl of medium under paraffin oil in a 35 × 10 mm tissue culture dish (Falcon Plastics, Oxnard, CA) at 37°C in an atmosphere with 5% CO_2 for 16 to 18 h. Ten to 15 complexes were present in each drop. To prepare denuded oocytes, the surrounding cumulus cells were removed by passing them through a fine flame mouth-operated micropipette. The denuded oocytes were washed and incubated in the same manner as the cumulus-oocyte