Inhibition of Cell Division in Mammalian Cell Cultures by Hypertonic medium

The arrest of cells in metaphase by hypertonicity has previously been described for chick cells by HUGHES[1] who noted that further entry of cells into division was prevented. STUBBLEFIELD and MUELLER[2] found that increasing the tonicity of culture medium with NaCl arrested some HeLa cells in metaphase, with a small accumulation of mitoses occurring over a 24 h period. This suggests a reduced rate of cell cycling with a preferential inhibition in mitosis. Recent evidence[3,4] has suggested that hypertonic conditions produce a prophase-like appearance in HeLa cells with macromolecular syntheses being reduced in a similar manner to that seen when cells normally enter mitosis. Our experiences with hypertonicity on the entry of cells into mitosis are outlined here; detailed reports will be published later.

Tonicity and mitotic arrest in HeLa and other cell lines. HeLa S-3 cells in monolayer and suspension culture[5] were grown in Eagle's HeLa medium of about 165 m*M* strength. The tonicity was raised by the addition of calculated amounts of 20 × concentrated Earle's BSS or saline which gave identical results. Growth of HeLa S-3 in 206 m*M* medium was slightly depressed; at 227 m*M* growth was 40 % of the control rate and at 240 m*M* cell division and death were in equilibrium over a 3-4 day period. These results agree with previous reports[2,6].

There was metaphase arrest of cells at a medium strength of 215 m*M* and above, being most evident between 220 and 240 m*M*. Above 240 m*M* pyknotic changes became increasingly pronounced in both mitotic and interphase cells. At 227 m*M* chromosomes initially appeared to be more heavily condensed than normal but there was a well-defined spindle (Figure 1). Electron microscopic examination showed these mitoses to be largely indistinguishable from normal metaphases (Figure 2), including the spindle structure. Hypertonically-induced metaphase arrest is not, therefore, due to lack of spindle assembly. Metaphase cells held for 2–3 h at 227 m*M* show definite anaphase movements of individual or groups of chromosomes (Figure 1).

There was little evidence of prophase-like cells at optimal metaphase arresting tonicities. However, we have observed transient granularity in nuclei of HeLa cells 5 to 15 min after exposure to 290 m*M* medium (1.7 × isotonicity). One might confuse altered interphase nuclei with early prophases, but no definite prophases were 'induced'.

CHO-10 (Chinese hamster ovary) fibroblasts required a greater degree of hypertonicity than HeLa to arrest metaphases, usually about 265 m*M*, which allowed a small accumulation of metaphases with time. BHK/C13/DWS-3, suspension-adapted subclone of BHK21/C13 fibroblasts grown under identical conditions to HeLa S-3, also proved relatively resistant to hypertonic medium. A small amount of accumulation of metaphases was seen over 30-60 min at 290-300 m*M*. Obviously there are considerable differences in responses of cells to hypertonicity.

Hypertonicity and different cautions. K^+ (as KCl) arrested metaphases when added to bring the strength of normal medium to 200-215 m*M*. Li^+-induced arrest was pronounced at 200 m*M* and there was no escape of cells through anaphase. Both Ca^{++} and Mg^{++} were excellent blockers at 215 m*M* and 200 m*M* respectively. Small increases above these levels quickly produced toxic effects.

Entry into mitosis. HeLa S-3 grown in asynchronous monolayer cultures were exposed to 200 and 227 m*M* medium for 8 h. The result of a typical experiment is shown in Figure 3, in which it can be seen that 227 m*M* medium permitted entry of cells into mitosis at a normal rate for about 3 h when compared with a colcemid control. After 3 h there was variation from one experiment to another; sometimes continued slow metaphase accumulation occurred, or more often a fall off in metaphase index was seen. This was mainly due to varying degrees of progression out of mitosis. There was also evidence that S

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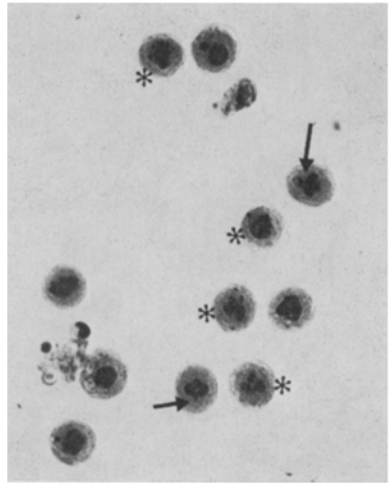

Fig. 1. Collection of mitotic cells from a culture of HeLa S-3 cells exposed to 227 m*M* medium for 3 h. Note the evidence of spindles in the cells given asterisks and the tendency of some chromosomes to move poleward in an attempted anaphase configuration (arrows). Crystal violet. × 385.

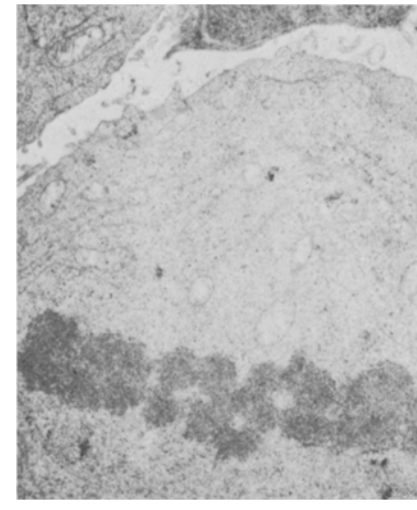

Fig. 2. Electron micrograph of a HeLa S-3 cell arrested in metaphase by 227 m*M* medium for 3 h. Glutaraldehyde fixed, Epon embedded, uranyl acetate/lead citrate staining. × 9,000.

phase cells progressed into metaphase more slowly in 227 mM medium as shown by combined treatment of hypertonic medium and colcemid (Figure 3). Thus G_2 cells are relatively insensitive to 227 mM medium while S phase cells are definitely retarded. Hypertonicity is reported to suppress DNA synthesis[3,7], and our data (to be published) confirm and extend this observation.

Reversibility of arrested metaphases. Metaphase cells were collected by mechanical agitation[8] from large monolayers of HeLa S-3 exposed to 227 mM medium for 3 h. Exit of cells from mitosis on resuspension in 165 mM medium was as rapid as for isotonically collected controls. Figure 4 shows the increase in cell number on reversal compared with unreversed cells which slowly fell in numbers over the recovery phase. Mitotic index at collection falls rapidly towards zero as for the isotonic controls both giving a second wave of mitosis between 22 and 31 h with co-incidental peaks at 23-25 h (Figure 5). Therefore hypertonically collected metaphases move out of mitosis synchronously and reach their next division without delay. Less reliable recovery occured with metaphases collected for more than 4 h in 227 mM medium.

Isotonic metaphases exposed to 227 mM medium immediately upon collection showed an insignificant fall in the metaphase index of the population over 6 h indicating an immediate arrest at this stage. Anaphases and early telophases progressed out of mitosis with difficulty, some showing failure to decondense their chromatin and becoming pyknotic. Cells initially in mid or late telophase progressed into interphase and produced a high percentage of binucleate-like cells due to nearly equal division of the nuclear mass (Figure 6). This occurred in cells which had successfully completed a normal cytokinesis shortly beforehand. These 'binucleate' cells often went on to produce more lobulations to their nuclei. This phenomenon of nuclear schism in early interphase was also seen in hypertonically collected metaphase cells shortly after reversal in isotonic medium.

Synchronization enhancement by hypertonic treatment. A population of HeLa cells exposed to 227 mM medium for 3 h allowed the collection of about 3 h worth of mitoses; the average yield for several experiments was 2.8 × that obtained by a 1 h colcemid collection. The population of mitotic cells was purer than from isotonic collection, rarely giving less than 98–99% mitotic cells, almost entirely comprised of metaphases. Since these cells readily progress into interphase in 165 mM medium, an improvement on shake-off techniques of synchronization[8] can be achieved especially as an alternative to colcemid enhancement procedures.

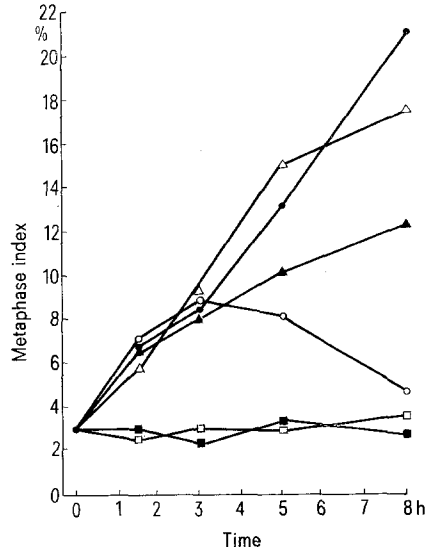

Fig. 3. Metaphase indices of suspension cultures of HeLa S-3 cells exposed to hypertonic medium with or without colcemid (1.5×10^{-7} M). □—□, isotonic (165 mM) medium; ●—●, isotonic medium + colcemid; ■—■, 200 mM; △—△, 200 mM + colcemid; ○—○, 227 mM; ▲—▲, 227 mM + colcemid.

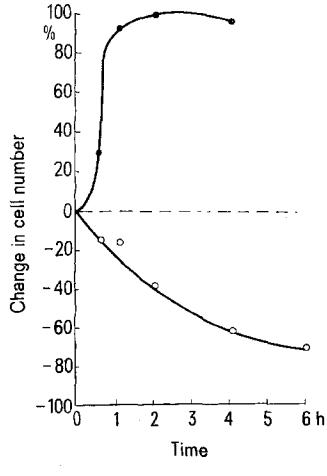

Fig. 4. Change in cell number estimated by haemocytometer counts of cells collected at 0 h from a culture made hypertonic (227 mM) 3 h previously. ●—●, mitotic cells resuspended in isotonic medium; ○—○, mitotic cells resuspended in 227 mM hypertonic medium.

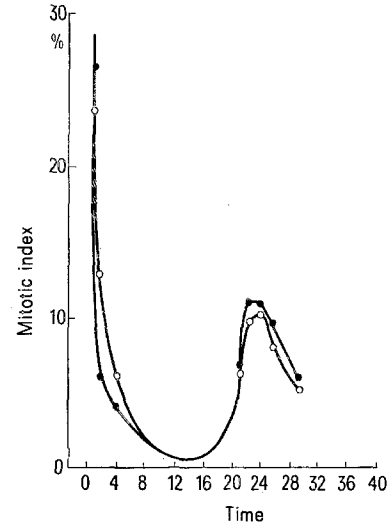

Fig. 5. Mitotic activity of cultures over 30 h after collection of mitotic cells at 0 h by mechanical agitation into normal medium. Cells collected from ○—○, isotonic culture (93% mitotic cells at 0 h); and ●—●, hypertonic 227 mM culture (97% mitotic cells at 0 h).

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Comment. Raised medium strength traps HeLa cells in metaphase while allowing a normal G_2 population to enter mitosis and retarding progression of S phase cells. This contrasts with HUGHES'[1] finding in chick cells where progression was immediately halted. At the levels of hypertonicity employed (about 227 m*M* for HeLa cells) there was no evidence of prophase 'induction' or nuclear chromatin condensation[3]. Cells progressed normally through this stage of mitosis in expected numbers.

It has been clearly demonstrated that hypertonically collected metaphases remain readily reversible for 3–4 h but degenerate quickly if held for longer periods of time. Hypertonic treatment induces an immediate metaphase arrest, but, isotonically collected anaphases and telophases in hypertonic medium (and hypertonically collected metaphases returned to isotonic medium) show unusual nuclear behaviour after completing normal cytokineses which involves the lobulation of the nucleus often into 2 roughly equal parts before further lobulation.

Synchronization of cells by mechanical collection in metaphase[8] is aided by the technique of hypertonic treatment and gives good yield of nearly pure metaphase cells. At present the cause of metaphase arrest by hypertonicity is unknown. There is no evidence of spindle abnormalities but chromosomes tend to be supercondensed and more sticky, probably preventing their anaphase separation.[10]

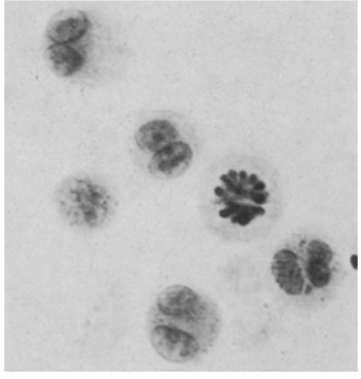

Fig. 6. Isotonically collected mitotic HeLa cells exposed to 227 m*M* hypertonic medium for 6 h. The metaphase shows heavy condensation of cromosomes into several groups. Cells which have reached interphase appear binucleate. Crystal violet. ×600.

Résumé. L'arrêt de la métaphase se produit dans les cellules de HeLa S-3 au contact d'un milieu de tonicité accrue (> 165 m*M*). L'accumulation optimale intervient à 277 m*M* lorsque du NaCl a été utilisé pour augmenter l'influence du milieu. Les effets de l'usage d'autres sels et d'autres types de cellules sont décrits. La dilution du milieu à 165 m*M* donne lieu à un arrêt synchrone du processus de la mitose dans les cellules et assure la conservation d'un bon synchronisme au cours de la division cellulaire suivante.

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10 April 1973.

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[10] This work was supported by the Cancer Research Campaign and the Wellcome Foundation. Mrs. M. INGLIS and Miss W. ELMSLIE provided technical assistance.

A New Liver-Specific Autoantigen in the Rat: Delta Antigen

When inbred mice were immunized with allogeneic liver extracts, autoantibodies reacting with the liver-specific 'F antigen' were induced in certain strain combinations[1,2]. Since it seemed unlikely that the F antigen system reflected a situation unique to the mouse, we investigated the capacity of inbred rats to be similarly immunized.

Rats from inbred strains DA, Lewis and BN were used. Aqueous liver extracts from each strain were emulsified in complete Freund's adjuvant and injected every other week into syngeneic or allogeneic rats by the intraperitoneal route. Up to 5 injections were given. Serum samples were tested at intervals for the presence of precipitating antibodies by the Ouchterlony technique, liver extracts from each of the three strains serving as antigens.

Table I. Results of immunization of rats with rat liver extracts

Strain immunized	Stimulating liver extracts from DA	Lewis	BN
DA	− −	− −	− −
Lewis	**	− −	**
BN	**	+ +	− −

− −, No precipitating antibody detectable; **, weak precipitating alloantibody not reacting with Lewis extract (see text); + +, strong precipitating autoantibody reacting with all 3 rat liver extracts and defining the liver antigen Delta.

The results of these immunizations are shown in Table I. As in the mouse, even prolonged syngeneic stimulation (e.g., BN liver extracts injected into BN rats) never yielded precipitating antibodies. DA rats proved also refractory to allogeneic stimulation by either Lewis or BN extracts; this was reminiscent of the situation in BALB/c and DBA/2 mice which did not make precipitating antibodies upon stimulation with several mouse liver extracts[1]. Lewis rats, after immunization with either DA or BN extracts, did produce weak precipitating antibodies; these antibodies, however, were not autoantibodies, since they failed to precipitate syngeneic (Lewis) extracts. Rather, they appeared to recognize some alloantigen characteristic of strains DA and BN, and different from known rat allotypes. This system, for which no counterpart in the mouse is known, has not been studied in detail.

BN rats, upon stimulation with Lewis antigen, after 3 to 4 injections regularly produced strong precipitating antibody which reacted with all 3 liver extracts and had

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