Effects of 5-Bromo-2'-deoxyuridine on Beating Heart Cell Cultures from Neonatal Hamsters

By GERHARD A. COETZEE, DENEYS R. VAN DER WESTHUYZEN
and WIELAND GEVERS

M.R.C. Research Unit for Molecular and Cellular Cardiology, University of Stellenbosch Medical School, P.O. Box 63, Tygerberg 7505, Cape, South Africa

(Received 30 November 1976)

1. Primary heart cell cultures from neonatal hamsters yielded a heterogeneous cell population, containing muscle cells undergoing progressive differentiation, as well as non-muscle cells. 2. Addition of 5-bromo-2'-deoxyuridine, at an early stage, to such cultures enhanced the formation of beating sheets of differentiated muscle cells. Accumulation of myosin heavy chains and creatine kinase also occurred in the presence of the analogue. 3. To obtain these effects, the analogue had to be added during the initial rapid growth phase of the cells. Division of the treated cells then ceased when the cell numbers had approximately doubled. 4. Similar results were obtained with other inhibitors of DNA synthesis. Thus improved muscle cell cultures can be obtained by preventing non-muscle cells from overgrowing the cultures. 5. One effect caused only by 5-bromo-2'-deoxyuridine was a large increase in the Ca^{2+} -stimulated ATPase (adenosine triphosphatase) activity which sedimented at low ionic strength. This increase was not due to a greater content of myofibrillar myosin, or to myosin isoenzyme changes, because purified myosin prepared from treated and untreated cultures did not exhibit the increased Ca^{2+} -stimulated ATPase activity.

The thymidine analogue BrdUrd* has marked modulating effects on the expression of differentiated properties of cells in culture (Rutter *et al.*, 1973). Generally, these effects are inhibitory in nature. For example, the incorporation of BrdUrd into the DNA of skeletal myoblasts results in the failure of these cells to differentiate, as shown by the absence of both cell fusion and the synthesis of myofibrillar myosin (Bischoff & Holtzer, 1970; Mochan *et al.*, 1974). The analogue also adversely affects the accumulation of tissue-specific proteins by pancreatic cells (Walther *et al.*, 1974), hepatoma cells (Stellwagen & Tomkins, 1971), retina cells (Zimmerman *et al.*, 1974), chondrocytes (Schulte-Holthausen *et al.*, 1969; Chacko *et al.*, 1969) and primitive erythroblasts (Weintraub *et al.*, 1973).

In some terminally differentiated but mitotically active cells, however, the incorporation of BrdUrd into DNA has no effect on the particular gene-expression characteristic of the differentiated state (Weintraub *et al.*, 1973). Such a situation seems also to exist in cardiomyocytes, since the differentiation of

embryonic-chick heart cells *in vivo* becomes asynchronously resistant to BrdUrd at about stage 7 of embryonic development. Cell cultures prepared from hearts which are further developed are also resistant to the modulating effects of BrdUrd, as assessed by the amount of fluorescein-labelled myosin-specific antibody bound per cell (Chacko & Joseph, 1974).

In a study where the gene products of chick chondroblasts dividing in a culture in the presence of BrdUrd were analysed, the nucleoside interfered with the regulation of gene expression in a manner more complex than simple suppression of the synthesis of all specialized cell products (Mayne *et al.*, 1975): a switch occurred in the predominant type of collagen chains synthesized by the chondroblasts. Also, Schubert & Jacob (1970) have demonstrated an enhancing effect of BrdUrd addition on the expression of certain differentiated functions by cultured neuroblastoma cells, not involving its incorporation into DNA. In addition, BrdUrd induces variations in the activities of alkaline phosphatase in several human heteroploid cell lines (Bulmer *et al.*, 1976).

The effects of BrdUrd so far observed on differentiating or differentiated cells in culture are thus

* Abbreviations: BrdUrd, 5-bromo-2'-deoxyuridine; FdUrd, 5-fluoro-2'-deoxyuridine; SDS, sodium dodecyl sulphate; ATPase, adenosine triphosphatase (adenosine triphosphate γ -phosphohydrolase, EC 3.6.1.25).

diverse and no generalized concept has emerged, although, with the *lac* operon, defective regulation at the molecular level of repressor interaction with BrdUrd-containing DNA has been defined *in vitro* (Lin & Riggs, 1974). It was also noted by Strom & Dorfman (1976) that BrdUrd is preferentially incorporated into moderately repetitive regions of mesenchyme cellular DNA; these are thought to be 'address sites' on the DNA to which regulatory substances may bind to modulate gene activity. The mutagenic effect of BrdUrd, especially when treatment is accompanied by exposure to visible light, may also be the cause of altered cellular function (Davidson & Bick, 1974; Masse & Harary, 1974; Bick & Soffer, 1976). The many diverse effects of BrdUrd cannot, however, all be explained by its incorporation into DNA, and alternative mechanisms have been proposed (Schubert & Jacob, 1970; Meuth & Green, 1974; Rogers *et al.*, 1975).

In the present paper, we describe a biochemical analysis of the behaviour of cultured neonatal-hamster heart cells treated with BrdUrd. A relatively large number of tissue-culture systems for the study of chicken, rat and hamster cardiomyocytes have previously been described (reviewed by Zak, 1973). Morphological differentiation of cultured chicken cardiomyoblasts (Halle & Wollenberger, 1970; Chacko, 1973) and neonatal-rat heart cells (Halle & Wollenberger, 1970) has been observed. However, biochemical studies on such systems as those of Harary *et al.* (1963) and Ziter (1974) are complicated by the presence of non-muscle cells, which have been described as fibroblast-like cells (Polinger, 1970, 1973; Chacko & Joseph, 1974) and endothelial cells (Kasten & Yip, 1974). Although cardiac muscle cells appear to be non-dividing in some systems (Halle & Wollenberger, 1970; Wollenberger *et al.*, 1972), definite evidence for division at a lower rate than in non-muscle cells has been obtained by others (Chacko, 1973; Polinger, 1973). Cardiac muscle cells are therefore fairly quickly overgrown in cultures by non-muscle cells (Polinger, 1973), although such overgrowth can be prevented by treatment of cultures with the mitotic inhibitor 5-fluorodeoxyuridine (Karsten *et al.*, 1973; Wollenberger *et al.*, 1972) and by glutamine deprivation (Clark, 1976).

In an attempt to analyse differentiative properties of neonatal-hamster hearts, we treated cultured cells from such hearts with BrdUrd. When added during the initial rapid proliferative phase of these cells, BrdUrd did not suppress, but actually promoted, the formation of a beating sheet of differentiated heart cells, but it prevented the attainment of high cell densities, and caused a large increase in the Ca^{2+} -stimulated ATPase activity. Unexpectedly, the Ca^{2+} -stimulated ATPase activity affected by the nucleoside treatment resided in cellular components unrelated to myofibrillar myosin.

Experimental

Materials

Syrian hamsters were maintained by random inbreeding in our colony obtained from the London School of Hygiene. Calf serum was prepared from pooled calf blood obtained from the local abattoir. Trypsin (1:250, Difco-certified) and tryptose/phosphate broth were obtained from Difco Laboratories, Detroit, MI, U.S.A. Eagle's minimum essential medium buffered with Earle's salts (Eagle, 1959) was from Grand Island Biological Co., Grand Island, NY, U.S.A., and penicillin G and streptomycin were from Hoechst Pharmaceuticals, Johannesburg, South Africa. Plastic tissue-culture dishes were obtained from Falcon, Division Becton, Dickinson and Co., Oxnard, CA, U.S.A. BrdUrd was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., and ATP (disodium salt), dithiothreitol and 2-mercaptoethanol were from Miles Laboratories, Goodwood, South Africa. [γ - ^{32}P]ATP was from The Radiochemical Centre, Amersham, Bucks., U.K. Instagel was from Packard Instruments, Downer's Grove, IL, U.S.A. Polyacrylamide and SDS were from BDH Chemicals, Poole, Dorset, U.K., and Sepharose 4B was from Pharmacia, Uppsala, Sweden. Other reagents were from E. Merck A.G., Darmstadt, Germany. The reagents were of analytical grade or of the highest purity available.

Primary heart cell cultures

Cultures were established under aseptic conditions as described by Harary & Farley (1963), with a number of modifications. Hearts from 30–70 Syrian hamsters (1–2 days old) were cut into cubes, about 0.5 mm thick, which were washed for 10 min in a 'trypsinizing flask' with 25 ml of phosphate-buffered saline containing no Ca^{2+} and Mg^{2+} (Dulbecco & Vogt, 1954). After decantation of the saline, 25 ml of phosphate-buffered saline containing 0.1% trypsin was added, and stirring was continued at 30°C for 10 min. Cells liberated in this way were discarded. The latter treatment was repeated, and liberated cells were again discarded (Kasten, 1973). The tissue was similarly treated with trypsin for two further periods of 30 min each, and in each case the disaggregated cells were collected by centrifugation at 1000g for 2 min. They were then suspended in 10 ml of medium (Eagle's minimum essential medium, buffered with Earle's salts and containing 10% tryptose/phosphate broth, 10% calf serum, inactivated at 56°C for 30 min, 60 μg of penicillin G/ml and 100 μg of streptomycin sulphate/ml).

The suspended cells were filtered (no. 7 nylon mesh, 36 divisions/cm) and the viable cells (usually about 70%) were assessed by Trypan Blue exclusion. Cells were seeded as a routine at 2.0×10^6 viable cells/5 ml of medium in Falcon (60 mm diameter)

plastic dishes and incubated at 37°C in an atmosphere of air/CO₂ (19:1). The medium was changed every second day, after unattached cells and debris had been washed off with phosphate-buffered saline. BrdUrd in phosphate-buffered saline was added to certain cultures on day 1 to give a final concentration of 0.1mM. Unless otherwise specified, BrdUrd was then maintained at the same concentration in all subsequent changes of medium of treated cultures. Cells treated with BrdUrd were incubated in the dark and kept out of direct light during changes of medium. Cultures were observed daily on the stage (heated to 37°C) of an inverted Nikon microscope equipped with phase-contrast optics. For staining purposes, attached cells were washed with phosphate-buffered saline, fixed with 10% phosphate-buffered formalin, and stained with phosphotungstic acid/haematoxylin (Lillie, 1954). Cells were examined and photographed with a Nikon camera with Ilford FP4 film (ASA 125).

Harvesting, counting and homogenization of cultured cells

Attached cells were harvested by treatment for 10min with 2ml of phosphate-buffered saline, containing 0.1% trypsin, at room temperature (25°C). They were then scraped off with a rubber policeman, washed with 3×10ml of medium and 1×10ml of phosphate-buffered saline, and counted. This procedure was adopted to facilitate counting of cells, and the treatment made no significant difference to subsequent enzyme assays.

Cells (usually pooled from two or three dishes) were suspended in 200μl of buffer H (10mM-Tris/HCl/1mM-EDTA/1mM-dithiothreitol, pH7.4) and frozen in liquid N₂. Frozen cells were allowed to thaw and swell in 0.4ml of buffer H for 1h at 4°C. Homogenization was effected with 60 passes of a tight-fitting Dounce homogenizer (Blaessig Glass, Rochester, NY, U.S.A.).

Enzyme assays

All homogenates were centrifuged at 10000g for 10min and the supernatants were used for the determination of the activities of creatine kinase (Oliver, 1955) (EC 2.7.3.2) and lactate dehydrogenase (Bergmeyer *et al.*, 1963) (EC 1.1.1.27); such supernatants contained 80–100% of the total cellular activities. The sediments containing particulate myosin (Solaro *et al.*, 1971) were quantitatively extracted by suspension in 0.1ml of 0.6M-KCl/1mM-ATP/1mM-dithiothreitol, pH7.0, followed by frequent mixing at 0°C for 60min, before centrifugation in the cold at 10000g for 10min. No myosin heavy chains were detected on SDS/polyacrylamide-gel electrophoretic analysis of the residual pellet, and no myosin ATPase activity was liberated by re-extraction of this pellet.

The Ca²⁺-stimulated ATPase activity was measured at 25°C by determining the release in 10min of ³²P from [γ-³²P]ATP, in mixtures (100μl) containing 50mM-Tris/acetate, 10mM-CaCl₂, 0.12M-KCl, 1mM-EDTA, 1mM-EGTA and 1mM-[γ-³²P]ATP (0.5μCi), all at pH7.4, and linearly limiting amounts of enzyme. For (K⁺+EDTA)-dependent ATPase activities [(K⁺+EDTA)-ATPase], the same conditions were used, except that 0.62M-KCl with no CaCl₂ was present. After the incubation, samples were immediately mixed with 4vol. of 2.5% (w/v) acid-washed Norit A in 2% (v/v) HClO₄ also containing 0.05M-NaH₂PO₄, and centrifuged at 15000g in a Beckman Microfuge. Radioactivity in 25μl samples of the supernatants was determined by liquid-scintillation counting in 5ml of Instagel. Dependence of activity on Ca²⁺ was almost total, in that omission of Ca²⁺ and inclusion of 5mM-EGTA eliminated 95% of the activity. Oligomycin-sensitivity was absent. P_i was the only radioactive product of the reaction, as indicated by high-voltage electrophoresis of deproteinized extracts (Jones *et al.*, 1976). This highly sensitive and rapid assay procedure gave values similar to those obtained by the colorimetric method of determining P_i (Fiske & Subbarow, 1925). Protein concentrations were determined colorimetrically with bovine serum albumin as standard (Lowry *et al.*, 1951). All enzyme units are defined as 1nmol of substrate utilized/min under the conditions given.

Polyacrylamide-gel electrophoresis in the presence of SDS

Samples were dialysed for 2h against 200vol. of 6M-urea, containing 1% 2-mercaptoethanol and 0.075M-sodium phosphate, pH7, at room temperature. SDS was then added to give a final concentration of 1%. Electrophoretic separation of proteins on SDS-containing 5% (w/v) polyacrylamide gels (Paterson & Strohmman, 1970) was followed by staining with Coomassie Brilliant Blue (Weber & Osborn, 1969). A myofibrillar preparation from neonatal-hamster hearts was prepared (Solaro *et al.*, 1971) and analysed to identify myofibrillar proteins. Purified adult-hamster heart myosin (Fenner *et al.*, 1973) and actin (Spudich & Watt, 1971) were also used as standards. Gels were scanned with a Vitatron TLD 100 densitometer. The relative amounts of myosin heavy chains were determined by integrating the myosin heavy-chain densitometric peaks. This was done only when the relative colour yield of myosin heavy chains was linearly proportional to the protein concentration of the starting material.

Purification of myosin from cultures

Homogenates of cells were centrifuged at 10000g for 10min at 4°C. The pellets obtained were homo-

genized with five passes of a tight-fitting Dounce homogenizer in 1 ml of 0.6M-KCl/1mM-ATP/1mM-dithiothreitol, pH 7.0, and actomyosin was extracted at 0°C for 60 min with frequent stirring. Re-extraction tests showed that no myosin remained in the residues after re-centrifugation as above. The supernatants obtained were dialysed for 6 h at 4°C against 500 vol. of the original homogenization buffer, to allow reprecipitation of myosin. The precipitates were collected by centrifugation as above, suspended in the original extraction medium, cleared by centrifugation as above and reprecipitated by dialysis as above. The precipitation cycle was repeated twice

more. The final precipitates were subjected to the special gel-filtration step (Sephacrose 4B) described by Pollard *et al.* (1974), involving a two-buffer system designed to minimize exposure of myosins to deleterious effects of high concentrations of KI.

Results

About one-quarter of the 2 million viable cells seeded on each dish attached within 12 h and the number of cells then increased. When BrdUrd (0.1 mM) was added to such cultures 1 day after plating, it prevented the normal rapid proliferation of cells seen in untreated cultures between days 2 and 3 (Fig. 1). However, the cells in BrdUrd-treated cultures apparently underwent one division in the presence of the nucleoside, since the cell number almost doubled between day 1 and day 2. Removal of the base analogue on day 3, by addition of fresh medium after one rinse, did not result in a subsequent increase in cell number, showing that the mitotic block could not be reversed to a significant degree.

Individual contracting cells were observed in all cultures during the first 1 or 2 days, most of them beating asynchronously. Later, more cells became active, forming groups of synchronously beating cells. Finally, on days 4 and 5, the cells in areas of untreated cultures were observed to beat as synchronous 'sheets', although, as noted by Polinger (1973), actively contracting and interconnected passively moving cells in such groups or 'sheets' could not easily be distinguished. These cultures, however, also contained many areas of non-beating cells. BrdUrd-treated cultures, on the other hand, consisted of a less-dense cell layer with more actively beating foci.

At least two kinds of cells could easily be distinguished when cultures were stained with phosphotungstic acid/haematoxylin. This procedure helps

Fig. 1. *Proliferation of heart cells in culture*
Cells were seeded at 2.0×10^6 /dish (●). BrdUrd was added on day 1 and kept thereafter at a concentration of 0.1 mM (○); BrdUrd-treated cultures, with BrdUrd removed on day 3 (△).

Table 1. *Enzyme activities in primary heart cell cultures*

Values are means of between four and ten separate experiments $\pm$ S.E.M. BrdUrd was added on day 1 at a concentration of 0.1 mM and maintained at this concentration in subsequent changes of medium. Analyses were performed as described in the Experimental section. All differences between untreated and treated cultures on day 5 were statistically significant (95% confidence level of paired *t* test), except for lactate dehydrogenase activities/cell.

Enzyme		Untreated		BrdUrd-treated	
		Day 2	Day 5	Day 2	Day 5
Ca ²⁺ -dependent ATPase	(units/10 ⁶ cells)	2.42 $\pm$ 0.55	3.36 $\pm$ 0.80	5.57 $\pm$ 2.05	15.7 $\pm$ 2.7
	(units/mg of protein)	7.8 $\pm$ 1.5	8.6 $\pm$ 2.4	15.9 $\pm$ 5.4	29.8 $\pm$ 6.0
Myosin heavy chain (relative to values for day 5 of untreated cultures)	(colour yield/cell)	0.32 $\pm$ 0.19	1	0.29 $\pm$ 0.20	1.90 $\pm$ 0.17
	(colour yield/mg of protein)	0.34 $\pm$ 0.23	1	0.35 $\pm$ 0.22	1.30 $\pm$ 0.07
Creatine kinase	(units/10 ³ cells)	0.129 $\pm$ 0.022	0.280 $\pm$ 0.066	0.250 $\pm$ 0.062	0.510 $\pm$ 0.106
	(units/ μ g of protein)	0.41 $\pm$ 0.07	0.73 $\pm$ 0.16	0.70 $\pm$ 0.12	0.91 $\pm$ 0.16
Lactate dehydrogenase	(units/10 ³ cells)	0.128 $\pm$ 0.022	0.327 $\pm$ 0.048	0.173 $\pm$ 0.022	0.329 $\pm$ 0.065
	(units/ μ g of protein)	0.43 $\pm$ 0.05	0.92 $\pm$ 0.14	0.48 $\pm$ 0.04	0.56 $\pm$ 0.12

to resolve internal structures (Chacko & Joseph, 1974). Striated myofibrils could be seen in some of the cells, and these were assumed to be muscle cells. At an early stage in culture the myofibrils were few in number, but later they were plentiful in BrdUrd-treated cultures. However, the myofibrils never took up parallel positions in register and the cells thus never attained the organizational level of their progenitors in 1-day-old hamster hearts. It was impossible to obtain clear stained preparations in untreated cultures when cells were incubated for longer than 3 days, because of the increased thickness of the cultures.

The question as to whether BrdUrd inhibited the expression of differentiated properties in our heart cell cultures was assessed by determining the activities of two groups of enzymes both generally considered to be especially prevalent in muscle cells. These were creatine kinase and lactate dehydrogenase activities as a reflexion of cytoplasmic energy metabolism, and the Ca^{2+} -dependent ATPase activity, combined with determination of total myosin heavy-chain content assessed on polyacrylamide gels, as guides to the contractile apparatus (Table 1). Untreated cultures displayed some degree of differentiation as assessed by these criteria. The measured Ca^{2+} -dependent ATPase activities did not correlate with the increases in myosin heavy-chain contents during the development of the cells in culture. BrdUrd treatment promoted the differentiation of muscle cells, since creatine kinase activities as well as myosin heavy-chain contents reached significantly higher values after 5 days in treated cultures than in untreated controls (statistically analysed by the paired *t* test). Lactate dehydrogenase activities, however, did not reflect this. The effect of BrdUrd on the Ca^{2+} -dependent ATPase activities was particularly pronounced, and 1 day after the beginning of BrdUrd treatment, treated cells contained twice the activity of untreated controls. This difference increased to 5-fold after 4 days of treatment. Such major differences between treated and untreated cultures were not observed for myosin heavy chains. The electrophoretic mobilities of myosin heavy chains

obtained from the two culture types did not differ (Fig. 2). However, myosin and actin were more predominant in crude extracts of cellular particulate material obtained from BrdUrd-treated cultures. It was impossible accurately to identify myosin light chains in the gels prepared from the crude extracts,

Fig. 2. Polypeptides separated by SDS/polyacrylamide-gel electrophoresis

Polypeptides were electrophoretically separated on 5% (w/v) polyacrylamide gels in the presence of SDS, and stained with Coomassie Blue. Densitometric scans were carried out at 550nm. (a) Myofibrillar extracts from untreated cultures (100 μg of protein); (b) myofibrillar extracts from BrdUrd-treated cultures (60 μg of protein). Myosin heavy chains and actin were identified by co-electrophoresis of purified standards obtained from adult-hamster hearts.

since many other unidentified polypeptides migrated in the same regions of the gels. Two conclusions can be drawn from these results: (1) BrdUrd did not adversely affect the differentiated state of the muscle cells in culture, and (2) Ca^{2+} -dependent ATPase activities did not correlate with myosin heavy-chain contents.

Since we had originally assumed that Ca^{2+} -dependent ATPase activity could be used as a quantitative marker for myosin, these discrepancies between myosin heavy-chain content and Ca^{2+} -dependent ATPase activity were further investigated in BrdUrd-treated and untreated cells.

Fig. 3. Ca^{2+} -dependent ATPase activities of heart cells grown in culture

Activities were determined in extracts obtained from cultures grown in the absence (●) and presence of 0.1 mM-BrdUrd added on day 1 (○), and were expressed as a function of total cellular protein.

The effects of BrdUrd on the Ca^{2+} -dependent ATPase activities of heart cell cultures were followed for 15 days in culture (Fig. 3). The progressive increase in the Ca^{2+} -dependent ATPase activity as a function of total cellular protein persisted for about 2 weeks in culture in the presence of BrdUrd, whereas the same activity increased only slightly after the first few days in untreated cells, after which it decreased rapidly. Untreated cultures deteriorated and started to peel away from the dishes after 10 days in culture, and longer incubations were not possible.

Addition of BrdUrd to cultures on days 1, 2, 3 and 4, and subsequent exposure for 4 more days, led to progressively smaller responses in terms of differential gains in Ca^{2+} -dependent ATPase activities relative to controls (results not shown). This suggests that mitotic activity at the time of nucleoside addition was necessary for the generation of the effect.

To ascertain whether the measured activities of Ca^{2+} -dependent ATPase were being influenced by the presence of an excess of exchangeable inhibitory or activating substances in the extracts, mixtures of extracts from the two culture types were also assayed. Such systems always exhibited the Ca^{2+} -dependent ATPase activities expected from simple mixtures of the two components, indicating that inhibitors or activators were not responsible for the differences.

Another possible explanation for the properties of BrdUrd-treated cells was that the analogue acted mainly or wholly by preventing the overgrowth of muscle cells by non-muscle cells (Polinger, 1973). Because myosins found in non-muscle cells generally have a lower specific ATPase activity than those found in white or red striated muscles (Pollard & Weihung, 1974), the lower Ca^{2+} -dependent ATPase activities in relation to the amount of myosin heavy chains present in untreated cultures could have been the result of higher relative contents of non-muscle myosins. BrdUrd-treated cultures were accordingly compared with cultures treated with well-known DNA-synthesis inhibitors, namely 1- β -D-arabinofuranosylcytosine (Cohen, 1967) and FdUrd (Heidelberg, 1965) (Table 2). Both inhibitors, when added

Table 2. Effect of BrdUrd, 1- β -D-arabinofuranosylcytosine and FdUrd on cell number, Ca^{2+} -dependent ATPase and myosin heavy-chain content as a function of total cellular protein

BrdUrd addition occurred 24h after seeding, and 1- β -D-arabinofuranosylcytosine and FdUrd were added 36h after seeding. The analogues were maintained throughout culture duration at indicated concentrations. Analyses were performed after cells were incubated for 10 days. Similar results were obtained in three separate experiments.

Treatment	$10^{-6} \times \text{No. of cells/dish}$	Ca^{2+} -dependent ATPase (units/mg of protein)	Heavy-chain content (colour yield /mg of protein, relative to value for untreated cells)
Untreated	4.0	3.8	1
0.1 mM-BrdUrd	1.0	20.0	1.32
0.01 mM-1- β -D-arabinofuranosylcytosine	0.9	9.0	1.45
0.1 mM-FdUrd	1.2	8.8	1.45

36h after the cells had been seeded to ensure equivalent cell densities, immediately prevented any further increase in cell numbers and yielded cultures comparable with BrdUrd-treated cultures with respect to cell density and beating efficiency. Although both 1- β -D-arabinofuranosylcytosine- and FdUrd-treated cells showed higher Ca^{2+} -ATPase activities as a function of total cellular protein, when compared with controls, the effects of BrdUrd were very much greater, both at early stages and after 10 days in culture. The amounts of sedimentable myosin heavy chains, also expressed in terms of total cellular protein, were not proportionately increased by BrdUrd and were very similar to those found in cultures treated with the other two mitotic inhibitors. Thus the relative increases in the heavy-chain contents of these treated cultures were probably due to the prevention of overgrowth, whereas the Ca^{2+} -dependent ATPase effect specific to BrdUrd was unrelated to this factor.

Because of the large number of myosin isoenzymes now known to exist in mammalian cells (Wikman-Coffelt *et al.*, 1975), it is reasonable to suspect that BrdUrd could influence genetic controls over the synthesis of these forms in cultures of heart cells. In this connexion one could note that a switch in isoenzyme type of myosin in cross-innervation experiments has been demonstrated in skeletal muscles (Weeds *et al.*, 1974). It was necessary to purify myosin from the cell extracts to test this possibility. Cultures were treated with 0.1mM-BrdUrd on day 1 and then exposed to the analogue for a further 4 days before harvesting. At least 10^8 cells were required for the subsequent purification. Other cultures were treated with 0.1mM-FdUrd as controls with similar cell densities. Material obtained by repeated precipitation cycles (Chi *et al.*, 1975) was subjected to gel filtration on Sepharose 4B columns. ATPase elution profiles for both cultures were similar to those of purified myosin prepared from neonatal-hamster hearts. Electrophoretic analysis of peak fractions on SDS/polyacrylamide gels (Fig. 4) revealed that myosin represented about 90% of the material obtained from both types of cultures. The heavy chains as well as two light-chain components have identical electrophoretic mobilities

Fig. 4. *Polypeptides separated by SDS/polyacrylamide-gel electrophoresis*

Polypeptides obtained by the column chromatography were electrophoretically separated on 5% (w/v) polyacrylamide gels in the presence of SDS, stained with Coomassie Blue and densitometrically scanned at 550nm. (a) Neonatal-hamster hearts; (b) BrdUrd-treated hamster heart cell cultures; (c) FdUrd-treated hamster heart cell cultures. MHC, Myosin heavy chain; MLC1 and MLC2, myosin light chains 1 and 2 respectively.

Table 3. *Cation-dependence of ATPase activity of homogenates and of purified myosins*

Homogenates were prepared as described in the Experimental section, and myosin was purified, in each case to the same degree of homogeneity, as shown in Fig. 4.

Starting material		Ca ²⁺ -dependent ATPase (units/mg of protein)	(K ⁺ +EDTA)- dependent ATPase (units/mg of protein)	Inhibition of Ca ²⁺ - dependent ATPase by 1 mM-Mg ²⁺ (%)
Homogenates	Neonatal-hamster hearts	54	28	83
	BrdUrd-treated cultures	60	9.8	19
	Fluorodeoxyuridine-treated cultures	18	5.2	18
Purified myosin	Neonatal-hamster hearts	323	233	90
	BrdUrd-treated cultures	590	381	85
	Fluorodeoxyuridine-treated cultures	431	218	80

in the two preparations. The contaminant in both cases appeared to be M-line proteins or C-protein (Offer, 1972). Comparison of the myosin preparations on the basis of various enzymic parameters revealed only small differences (Table 3). The increase in specific Ca²⁺-ATPase activity of myosin obtained from BrdUrd-treated cultures over that obtained from the FdUrd-treated culture did not account for the greatly increased activity observed in homogenates or in crude extracts of such cells. Both of the myosin preparations from cells cultured with nucleoside analogues were more active than myosin prepared from intact hamster hearts. It is noteworthy that myosin from cultured cells was invariably deficient in the heavier of the two light-chain components (the reason for this is not yet clear).

Since none of the hypotheses tested above could fully account for the enhancement of Ca²⁺-dependent ATPase activity observed in BrdUrd-treated cell extracts, non-involvement of myofibrillar myosin in the effect was strongly indicated. This was confirmed in the studies reported in the following paper (Coetzee & Gevers, 1977).

Discussion

The state of cellular differentiation (or specialization) in heart cell cultures was assessed by measuring the amounts of certain entities characteristically prevalent in muscle cells in relation to the number of cells in culture and to total cellular protein. When hamster cardiac cells were grown in culture, they began to beat and became progressively more differentiated when judged by these criteria. Cultures treated with BrdUrd contained many more cells which beat actively, and for much longer, than control cultures. Treated cells increased greatly in size and accumulated myofibrils, as assessed in phosphotungstic acid/haematoxylin-stained preparations. Creatine kinase activities and myosin heavy-chain concentrations were not decreased relative to controls, but in fact increased. The fall in lactate dehydrogenase activity seems to be related to the high concentrations of this enzyme in non-muscle

cells (Weidemann & Gevers, 1975); it is thus not a satisfactory marker for muscle cells in these cultures. Thus the differentiated properties of these cells were not adversely affected by BrdUrd. This is consistent with the view that cardiomyocytes became BrdUrd-resistant with respect to differentiated properties during embryonic development (Chacko & Joseph, 1974).

The possibility that BrdUrd counteracted a tendency for muscle cells to de-differentiate is less likely, as Polinger (1973) has shown that the characteristic decrease in the percentage of glycogen-containing muscle cells in heart cell cultures is caused by overgrowth phenomena and not by de-differentiation of cardiomyocytes. Increases in cell numbers in our cultures could also be stopped by addition of 1- β -D-arabinofuranosylcytosine and FdUrd. The improved cultures obtained in this way provided better control systems for comparison with BrdUrd-treated cultures and which were more amenable to biochemical analysis than untreated cultures, since these deteriorated and peeled away from dishes after incubation times of 10 days or longer.

The combined use of BrdUrd incorporation into cellular DNA and irradiation with visible light eliminated dividing cells from rat heart cultures (Masse & Harary, 1974). In their studies the cultures displayed a loss on Ca²⁺-dependent ATPase activities coupled with the elimination of the dividing cells.

The new effect of BrdUrd that we observed in our cultures was the increased Ca²⁺-dependent ATPase activity, which could not be explained on the basis of proportionally increased myosin concentrations or myosin catalytic-centre activities in the cells. This effect was exerted when BrdUrd was added during the proliferative phase of the cultures and was absent if treatment was delayed until the plateau phase of growth. This suggests that replicative incorporation of BrdUrd into DNA is required for these effects to be expressed. It is noteworthy in this context that BrdUrd treatment, started after 1 day in culture, caused a block in proliferation when the cells had approximately doubled in number. BrdUrd is not considered to be a very effective inhibitor of

DNA synthesis (Bischoff & Holtzer, 1970). However, in HeLa cells, the second division after BrdUrd addition is delayed, and only a small fraction of the cells can continue to divide indefinitely (Simon, 1961; Toliver & Simon, 1967).

There are a number of cellular Ca^{2+} -dependent ATPase systems, other than myofibrillar myosin, which could be affected by BrdUrd treatment of our cultured cells. In the following paper (Coetzee & Gevers, 1977), the entity affected by BrdUrd treatment of our cells is identified as a surface Ca^{2+} -dependent ecto(adenosine triphosphatase).

The South African Medical Research Council, Atomic Energy Board and the Harry Crossley Foundation are thanked for financial support. Mr. R. A. Dyer rendered valuable assistance.

References

- Bergmeyer, H. U., Bernt, E. & Hess, B. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 736–743, Academic Press, New York and London.
- Bick, M. D. & Soffer, M. (1976) *Nature (London)* **260**, 788–791.
- Bischoff, R. & Holtzer, H. (1970) *J. Cell Biol.* **44**, 134–150.
- Bulmer, D., Stocco, D. & Morrow, J. (1976) *J. Cell. Physiol.* **87**, 357–366.
- Chacko, S. (1973) *Dev. Biol.* **35**, 1–18.
- Chacko, S. & Joseph, X. (1974) *Dev. Biol.* **40**, 340–354.
- Chacko, S., Holtzer, S. & Holtzer, H. (1969) *Biochem. Biophys. Res. Commun.* **34**, 183–189.
- Chi, J. C. H., Rubinstein, N., Strahs, K. & Holtzer, H. (1975) *J. Cell Biol.* **67**, 523–537.
- Clark, W. A. (1976) *Dev. Biol.* **52**, 263–282.
- Coetzee, G. A. & Gevers, W. (1977) *Biochem. J.* **164**, 645–652.
- Cohen, S. S. (1967) *Prog. Nucleic Acid Res. Mol. Biol.* **5**, 1–88.
- Davidson, R. L. & Bick, M. D. (1974) in *Control of Proliferation in Animal Cells* (Clarkson, B. & Baserga, R., eds.), vol. 1, pp. 205–212, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Dulbecco, R. & Vogt, M. (1954) *J. Exp. Med.* **99**, 167–182.
- Eagle, H. (1959) *Science* **130**, 432–437.
- Fenner, C., Mason, D. T., Zelis, R. & Wikman-Coffelt, J. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3205–3209.
- Fiske, C. H. & Subbarow, Y. (1925) *J. Biol. Chem.* **66**, 375–380.
- Halle, W. & Wollenberger, A. (1970) *Am. J. Cardiol.* **25**, 292–299.
- Harary, I. & Farley, B. (1963) *Exp. Cell Res.* **29**, 451–465.
- Harary, I., Fujimoto, A. & Kuramitsu, H. (1963) *Natl. Cancer Inst. Monogr.* **13**, 257–275.
- Heidelberger, C. (1965) *Prog. Nucleic Acid Res. Mol. Biol.* **4**, 1–50.
- Jones, P. A., Baker, M. S. & Benedict, W. F. (1976) *Cancer Res.* **36**, 3789–3797.
- Karsten, U., Kössler, A., Janiszewski, E. & Wollenberger, A. (1973) *In Vitro* **9**, 139–146.
- Kasten, F. H. (1973) in *Tissue Culture, Methods and Applications* (Kruse, P. F. & Patterson, M. K., eds.), 1st edn., pp. 72–81, Academic Press, New York and London.
- Kasten, F. H. & Yip, D. K. (1974) *In Vitro* **9**, 246–252.
- Lillie, R. D. (1954) *Histopathological Techniques and Practical Histochemistry*, p. 537, McGraw-Hill, New York.
- Lin, S. & Riggs, A. D. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 947–951.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
- Masse, M. J. & Harary, I. (1974) *Biochimie* **56**, 1581–1585.
- Mayne, R., Vail, M. S. & Miller, E. J. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 4511–4515.
- Meuth, M. & Green, H. (1974) *Cell* **2**, 109–112.
- Mochan, B. S., Mochan, E. & De La Haba, G. (1974) *Biochim. Biophys. Acta* **335**, 408–423.
- Offer, G. (1972) *Cold Spring Harbor Symp. Quant. Biol.* **37**, 87–93.
- Oliver, J. T. (1955) *Biochem. J.* **61**, 116–122.
- Paterson, B. & Strohmman, R. C. (1970) *Biochemistry* **9**, 4094–4105.
- Polinger, I. S. (1970) *Exp. Cell Res.* **63**, 78–82.
- Polinger, I. S. (1973) *Exp. Cell Res.* **76**, 243–252.
- Pollard, T. D. & Weihung, R. R. (1974) *Crit. Rev. Biochem.* **2**, 1–65.
- Pollard, T. D., Thomas, S. M. & Niederman, R. (1974) *Anal. Biochem.* **60**, 258–266.
- Rogers, J., Stephen, K. C., Coulter, M. B. & Sanwal, B. D. (1975) *Nature (London)* **256**, 438–440.
- Rutter, W. J., Picket, R. L. & Morris, P. W. (1973) *Annu. Rev. Biochem.* **42**, 601–646.
- Schubert, D. & Jacob, F. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **67**, 247–254.
- Schulte-Holthausen, H., Chacko, S., Davidson, E. A. & Holtzer, H. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **63**, 864–870.
- Simon, E. H. (1961) *Virology* **13**, 105–118.
- Solaro, R. J., Pang, D. C. & Briggs, F. N. (1971) *Biochim. Biophys. Acta* **245**, 259–262.
- Spudich, J. A. & Watt, S. (1971) *J. Biol. Chem.* **246**, 4866–4871.
- Stellwagen, R. H. & Tomkins, G. M. (1971) *J. Mol. Biol.* **56**, 167–182.
- Strom, C. M. & Dorfman, A. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1019–1023.
- Toliver, A. & Simon, E. H. (1967) *Exp. Cell Res.* **45**, 603–617.
- Walther, B. T., Picket, R. L., David, J. D. & Rutter, W. J. (1974) *J. Biol. Chem.* **249**, 1953–1964.
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412.
- Weeds, A. G., Trentham, D. R., Kean, C. J. C. & Buller, A. J. (1974) *Nature (London)* **247**, 135–139.
- Weidemann, P. A. C. & Gevers, W. (1975) *S. Afr. Med. J.* **49**, 533–537.
- Weintraub, H., Campbell, G. Le M. & Holtzer, H. (1973) *Nature (London) New Biol.* **244**, 140–142.
- Wikman-Coffelt, J., Fenner, C., Smith, A. & Mason, D. T. (1975) *J. Biol. Chem.* **250**, 1257–1262.
- Wollenberger, A., Karsten, U. & Kössler, A. (1972) *Cardiology* **56**, 224–230.
- Zak, R. (1973) *Am. J. Cardiol.* **31**, 211–219.
- Zimmerman, J., Brumbaugh, J., Biehl, J. & Holtzer, H. (1974) *Exp. Cell Res.* **83**, 159–165.
- Ziter, F. A. (1974) *Exp. Neurol.* **43**, 539–546.