5-Bromo-2'-deoxyuridine-Stimulated Calcium Ion- or Magnesium Ion-Dependent Ecto-(Adenosine Triphosphatase) Activity of Cultured Hamster Cardiac Cells

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1. Treatment of hamster heart cells in primary culture with 5-bromo-2'-deoxyuridine resulted in the greatly increased activity of a particulate Ca^{2+} - or Mg^{2+} -dependent ATPase (adenosine triphosphatase). 2. 5-Bromo-2'-deoxyuridine exerted these effects only when it was incorporated into cellular DNA, and then in a concentration-dependent manner. 3. Serially replated cells contained less of the activity (expressed as a function of total cell protein) than did the primary cultures, but the stimulation caused by 5-bromo-2'-deoxyuridine addition was much greater. 4. The affected enzyme was apparently localized in the plasma membrane of the cells with its active centre exposed to the outer environment [ecto-(ATPase) dependent on Ca^{2+} or Mg^{2+}]. 5. The activity was unaffected by treatment with *p*-chloromercuriphenylsulphonate, ouabain and verapamil. 6. Ecto-(5'-nucleotidase) activity was not increased by 5-bromo-2'-deoxyuridine treatment of cells, and ecto-(*p*-nitrophenyl phosphatase) activity was only slightly enhanced.

BrdUrd* exerts diverse and sometimes apparently contradictory effects on many mammalian cells grown in culture. We have reported a further effect of BrdUrd on a Ca^{2+} -dependent ATPase activity in primary cultures of hamster heart cells (Coetzee *et al.*, 1977). This Ca^{2+} -dependent ATPase activity was not associated with myofibrillar myosin.

The total ATP-hydrolysing activity of mammalian cells represents a variety of components, of which most require MgATP as substrate. Notable exceptions to this rule are the Ca^{2+} -dependent ATPase activity of myofibrillar myosin in the absence of actin (Fenner *et al.*, 1973), and a number of as yet undefined Ca^{2+} -dependent ATPase activities associated with membrane preparations of mammalian cells (McNamara *et al.*, 1971; Oliveira & Holzhacker, 1974; Gimmel'-reikh, 1975; Garnett & Kemp, 1975; Garnett *et al.*, 1976). These enzymes may include ecto-(ATPases) with active centres localized on the outside of the plasma membrane of cells (De Pierre & Karnovsky, 1974a,b; Stefanovic *et al.*, 1974, 1976; Medzihradsky *et al.*, 1975), which can also utilize Ca^{2+} instead of Mg^{2+} as cation cofactor for activity. Certain Mg^{2+} -dependent ATPases are stimulated by low concentrations of Ca^{2+} , including the Ca^{2+} -ATPase of the sarcoplasmic reticulum (MacLennan *et al.*, 1972);

these systems, however, do not hydrolyse ATP in the absence of Mg^{2+} .

In the present paper we analyse the markedly stimulatory effects of BrdUrd on a membrane-associated Ca^{2+} - or Mg^{2+} -dependent ATPase activity in cultured heart cells. The activity of ecto-(5'-nucleotidase) is unaffected by the treatment. These results are notable, since surface membranes seem to be important in a number of BrdUrd-treated cell systems. BrdUrd causes increased flattening of heart muscle cells in culture (Chacko & Joseph, 1974), prevents the fusion of skeletal-muscle cells in culture (Bischoff & Holtzer, 1970; Rogers *et al.*, 1975) and brings about the formation of axon-like processes (neurites) in cultured neuroblastoma cells (Schubert & Jacob, 1970).

The cellular effects brought about by BrdUrd treatment have regularly been ascribed to DNA-linked mechanisms, because BrdUrd must be incorporated into DNA to produce its diverse effects (Rutter *et al.*, 1973). Schubert & Jacob (1970) and Rogers *et al.* (1975) have suggested, however, that some effects of BrdUrd may be mediated primarily at the level of the plasma membranes of cells in culture. As pointed out by Rutter *et al.* (1973), DNA-linked mechanisms are not ruled out in such systems, since a limited but critical incorporation of BrdUrd may well occur into certain DNA sequences. Here we describe experiments showing that BrdUrd must be incorporated into the DNA of cultured cells to exert its effects on ecto-(ATPase) activity.

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

Experimental

Materials

The materials used were as described in the preceding paper (Coetzee *et al.*, 1977). Cell types other than hamster heart cells were provided by Dr. P. A. Jones of this Department. Br[6-³H]dUrd was purchased from The Radiochemical Centre, Amersham, Bucks., U.K., ouabain (Strophanthin-G) from BDH Chemicals, Poole, Dorset, U.K., verapamil hydrochloride (Isoptin) from Knoll A.G. Chemical Works, Ludwigshafen, West Germany, and *p*-nitrophenyl phosphate from Sigma Chemical Co., St. Louis, MO, U.S.A.

Cultures

Primary heart cell cultures were established, cells harvested, counted and homogenized as described previously (Coetzee *et al.*, 1977). Secondary, tertiary and quaternary cultures were obtained from primary heart cell cultures by trypsin treatment of monolayers and replating them in series at cell concentrations of 0.5×10^6 cells per 60mm-diameter dish. Cells treated with BrdUrd were incubated in the dark and kept out of direct light during medium changes. Hamster fibroblasts were prepared from decapitated and degutted 10-day-old Syrian-hamster embryos; to obtain the cells called H1, they were transformed by treatment with 3-methylcholanthrene (Jones, 1972). Bovine lung fibroblasts were obtained in the same way as hamster fibroblasts, by using embryonic bovine lungs to prepare the cells. E-1 cells were epithelioid cells derived from embryonic bovine hearts. Rat heart cells were obtained from 2-4-day-old rats as described above for hamster heart cells. C6-2B cells were examples of a clonal cell line derived from a rat astrocytoma (Benda *et al.*, 1968); A(T1)C13 cells were from a hamster-embryo cell line transformed with 1- β -D-arabinofuranosylcytosine (Benedict *et al.*, 1975), and C3H/10T $\frac{1}{2}$ cells (Clone 8) were a clonal cell line derived from mouse-embryo cells (Reznikoff *et al.*, 1973). All these cells were seeded in recommended media, but growth during BrdUrd exposure was always conducted in the medium described above.

Assays

Ca²⁺-dependent ATPase activities were determined in cell-free preparations as described previously (Coetzee *et al.*, 1977). For (K⁺+EDTA)-dependent ATPase activities, the same conditions were used, except that 0.62M-KCl with no CaCl₂ was used. Ecto-(ATPase) activities were assayed under the conditions used by Stefanovic *et al.* (1974); liberated P_i was, however, detected radiometrically as in the Ca²⁺-dependent ATPase assay mentioned above.

For each determination, four independent measurements of the activity were performed which did not differ by more than 5% from each other.

5'-Nucleotidase activities (EC 3.1.3.5) on the outer surface of tissue-culture cells were assayed as described by Stefanovic *et al.* (1976). The hydrolysis of 5'-AMP by non-specific phosphatases was prevented by *p*-nitrophenyl phosphate. P_i liberated from 5'-AMP and *p*-nitrophenyl phosphate was determined on separate dishes and the difference was taken as the P_i enzymically released from 5'-AMP. P_i was determined by the method of Fiske & Subbarow (1925).

Protein concentrations were determined colorimetrically, with bovine serum albumin as a standard (Lowry *et al.*, 1951).

Chromatographic and electrophoretic procedures

Polyacrylamide-gel electrophoresis in the presence of SDS, was performed as described in the preceding paper (Coetzee *et al.*, 1977).

Gel filtration on Sepharose 4B was carried out as described by Coetzee *et al.* (1977).

The products of ecto-(ATPase) activity were analysed by using two systems, firstly, by applying samples to strips of DEAE-cellulose paper (Whatman DE-81) for ascending chromatography in 0.25M-triethylamine in 16% (v/v) acetic acid, pH 3.5, and secondly, by subjecting acid-deproteinized mixtures to high-voltage paper electrophoresis on Whatman 3MM paper (50V/cm) for 1h in 0.05M-sodium citrate, pH 5.0, on a cooled plate. In both procedures, ATP, ADP and AMP standards were analysed together with the samples and identified on the dried strips by u.v. light. The positions of ³²P_i and unchanged [γ -³²P]ATP were determined by the use of a Packard radio-chromatogram scanner, model 7201.

Radioautography

Cells were grown on glass coverslips in Petri dishes and exposed to 0.1 mM-Br[³H]dUrd (2 μ Ci/ml of medium). At appropriate times, the medium was removed and the cells were washed with new medium not containing BrdUrd; further growth was then permitted in BrdUrd-free medium. At the end of the experiment, coverslips containing cells were removed, washed with phosphate-buffered saline (Dulbecco & Vogt, 1954) and fixed in 99.5% (v/v) methanol for 10min. The cells were dried at room temperature (25°C) and stained with aceto-orcein (McManus & Mowry, 1960). The coverslips were then dipped in Ilford L4 emulsion and allowed to develop for 4 weeks in the dark. To obtain the number of grains per nucleus, the number of grains in at least ten nuclei (picked at random) were counted, and the mean value $\pm$ S.E.M. was used.

Results

The initial part of this work involved the demonstration that treatment of hamster heart cells in primary culture with the thymidine analogue BrdUrd caused a marked increase in the Ca^{2+} -dependent ATPase activity extractable from the readily sedimenting fraction of cell homogenates (Coetzee *et al.*, 1977).

When such extracts were passed through Sepharose 4B columns, two peaks of Ca^{2+} -dependent ATPase activity were obtained (Fig. 1a). The excluded peak (A) had Ca^{2+} -dependent ATPase but very little (K^{+} +EDTA)-dependent ATPase activity, whereas the second retarded peak (B) displayed both types of activities, and was eluted from the column at a position

corresponding exactly to that of myofibrillar myosin (Coetzee *et al.*, 1977).

The supernatant fractions obtained from the initial medium-speed centrifugation of newly homogenized cells contained about half of the total Ca^{2+} -dependent ATPase activity of the homogenates. When chromatographed on the same Sepharose column, a single sharp peak (C) of Ca^{2+} -dependent ATPase activity was also excluded from the column, corresponding in position to peak A in the other experiment (Fig. 1b). No (K^{+} +EDTA)-dependent ATPase activity was present. Comparison of the enzymic properties of the material present in peaks A and C, for untreated and BrdUrd-treated cultures, showed that BrdUrd treatment caused a marked increase in peaks A and C; these activities were not inhibited by Mg^{2+} (Table 1).

The activity in peak C was particulate and sedimented completely during centrifugation at 100000g for 3h. All the activity was recovered in the pellet, which also contained 5'-nucleotidase activity. Treatment of the sediment with Triton X-100 (0.2%) caused all the Ca^{2+} -dependent ATPase activity to disappear; a sample of pure myofibrillar myosin was unaffected by the same treatment (Katz, 1974; Yazaki & Raben, 1974). When the materials present in peaks A or C were analysed by SDS/polyacrylamide-gel electrophoresis, many unidentified bands were observed; one of them corresponded in mobility to myosin heavy chains, and was present in small amounts only. Even though precise densitometric comparisons were difficult, it was nevertheless clear that the large increases in Ca^{2+} -dependent ATPase activity caused by BrdUrd treatment were not accompanied by proportional increases in the density of the myosin-like bands.

Fig. 1. Sepharose 4B column chromatography of fractions prepared from homogenates of 5-day-old cultured hamster heart cells treated with BrdUrd

In (a) the material chromatographed was extracted with 0.6M-KCl/1 mM-ATP/1 mM-dithiothreitol from particulate fractions sedimented by centrifugation of homogenates at low speeds, whereas (b) involved analysis of the supernatant fractions. The Sepharose 4B column (44cm x 1cm; void volume 10ml) was equilibrated with KCl/ATP buffer (0.6M-KCl, 1 mM-ATP, 1 mM-dithiothreitol, 10mM-Tris/HCl, pH 7.4). The top 15% of the column bed volume was filled with KI/ATP buffer (0.6M-KI, 5 mM-dithiothreitol, 5 mM-ATP, 20mM-Tris/HCl, pH 7.4). The eluent was KCl/ATP buffer. Fractions (1ml) were collected and assayed for Ca^{2+} -dependent ATPase (●) and (K^{+} +EDTA)-dependent ATPase (○) activities. It was not possible to determine protein contents in the eluates from the column because of the limited amounts, and the presence of dithiothreitol in the samples.

Table 1. Ca^{2+} -dependent ATPase activities of high-molecular-weight fractions prepared from hamster heart cells treated (or not treated) with BrdUrd

The materials used were activity peak fractions prepared from cell extracts as described in Fig. 1; further details are given in the Experimental section and in the text. The activity of a sample of pure myofibrillar myosin was inhibited by 96% at the concentration of Mg^{2+} used.

	Peak	Ca^{2+} -dependent ATPase (units/mg of protein)	Inhibition of Ca^{2+} -dependent ATPase by 1 mM- Mg^{2+} (%)
Untreated cells	A	70	1
	C	424	1
BrdUrd-treated cells	A	284	2
	C	1093	1

Fig. 2. Ca^{2+} -dependent ecto-(ATPase) activities of replated hamster heart cells in culture, as a function of time in culture. BrdUrd (0.1 mM) was added on day 1 (○), and to a separate set of dishes, 1- β -D-arabinofuranosylcytosine (10 μM) on day 2 (●). The analogues were maintained in the respective media for the duration of the cultures. The media were changed every second day. Assays were carried out as described in the Experimental section.

Since treatment of cells with BrdUrd affected a Ca^{2+} -dependent ATPase activity apparently unrelated to myofibrillar myosin, it was necessary to test the effects of BrdUrd on non-muscle cells from hamster hearts in the absence of muscle cells rich in myofibrils. Selection for the mitotically more active non-muscle cells was achieved by replating primary heart cell cultures in four serial passages. After the second passage, no more beating cells were observed, and the monolayered cells had a uniform fibroblast-like appearance. When cells of this type were treated with BrdUrd during their proliferative phase, the cells became bigger and flatter (as seen by phase-contrast microscopy) and ceased to divide at lower cell densities than untreated cultures. Extracts were prepared from the high-speed sediments prepared from such homogenized cells, as well as from a similar number of untreated cells, and both were analysed on Sepharose 4B columns. A dramatic 9-fold enhancing effect of BrdUrd was evident when the Ca^{2+} -dependent ATPase activities in the excluded protein peaks were compared.

These experiments made it abundantly clear that the Ca^{2+} -dependent ATPase activity, mainly affected by BrdUrd, was present either in the form of protein

aggregates or in organelles. ATPase activities which act in the presence of Ca^{2+} (and the absence of Mg^{2+}) or in the presence of Mg^{2+} alone, are known to exist on the outside of plasma membranes of brain cells in culture (Stefanovic *et al.*, 1974, 1976). The activity of ecto-(Ca^{2+} -dependent ATPase) was accordingly assayed in culture dishes *in situ*. Heart cells in the fourth passage were used, and BrdUrd-treated cells were compared with cells treated with 1- β -D-arabinofuranosylcytosine. The latter were chosen as the appropriate controls because untreated overgrown cultures peeled away from the dishes when cultured for longer than 10 days, whereas cultures treated with 1- β -D-arabinofuranosylcytosine (when treatment was started 2 days after seeding) attained a cell density similar to that of the BrdUrd-treated cultures. The results obtained were expressed as ecto-(Ca^{2+} -dependent ATPase) activity (per mg of cellular protein), as a function of the age of the cultures (Fig. 2). Cultures treated with BrdUrd and maintained in the presence of this analogue displayed progressively more activity, so that after 2 weeks in culture there was about 10 times as much activity as at the beginning of the treatment or about five times as much as was ever attained in the presence of the other base analogue.

The ecto-(Ca^{2+} -dependent ATPase) activity was linear with time for incubation periods of up to 60 min. All the cells were still viable after completion of the assay, as assessed by their ability to exclude Trypan Blue. When cells were harvested with 0.1% trypsin and assayed as a cell suspension in a similar way, the same values for the ecto-(Ca^{2+} -dependent ATPase) activity were obtained, and the P_i released was proportional to the number of cells added to individual assays (standard curve). The breakdown products of the reaction were ADP and P_i , as shown by analysis of acid-deproteinized extracts on high-voltage paper electrophoretograms and ion-exchange paper chromatograms.

The apparent K_m values for ATP and Ca^{2+} were 0.15 mM and 0.3 mM respectively. The enzyme was unaffected by treatment with 0.1 mM-*p*-chloromercuriphenylsulphonate, 1 mM-ouabain and 0.1 mM-verapamil, an inhibitor of transplasmalemmal Ca^{2+} flow in cardiac cells (Kohlhardt *et al.*, 1972). When either Ca^{2+} or Mg^{2+} concentrations were varied in the assay, with the alternative bivalent cation present at a fixed concentration of 3 mM, the activity remained unchanged. It is difficult to be certain that traces of Mg^{2+} are not required for the activity of Ca^{2+} -dependent ATPase, or that two different entities may not be involved. These characteristics are very much the same as those observed by Stefanovic *et al.* (1976) for an ecto-(ATPase) on the cell surface of neuroblastoma cells in culture.

A number of different cell types were treated with BrdUrd, each on day 1, in culture to ascertain whether

Table 2. *Effects of BrdUrd treatment on ecto-(Ca²⁺-dependent ATPase) of various cell types grown in culture*
 BrdUrd (0.1 mM) was added 1 day after each seeding and (see the Experimental section) subsequently maintained in the media, which were changed every second day. Enzyme analyses were performed on day 5 of each culture, by using the assay described in the Experimental section. Abbreviation: ND, not detectable.

	Passage no.	Ecto-(ATPase) (units/mg of protein)		Ratio $\frac{\text{treated}}{\text{untreated}}$
		Untreated	BrdUrd-treated	
H1 (transformed hamster fibroblasts)	368	0.7	10	14.4
Hamster body fibroblasts	3	12	107	8.6
Hamster heart cells	4	41	347	8.5
A(T1)C13 (transformed hamster-embryo cell-line)	High	5	16	3.1
Primary hamster heart cells	—	91	217	2.4
Rat heart cells	3	69	148	2.1
E-1 (epithelioid bovine heart cells)	9	35	65	1.9
C3H/10T $\frac{1}{2}$ Clone 8 (mouse-embryo cells)	20	16	23	1.5
Primary chick skeletal-muscle cells	—	132	140	1.1
Bovine-embryo lung fibroblasts	3	ND	ND	—
C6-2B (rat astrocytoma cells)	26	ND	ND	—

Table 3. *Effects of BrdUrd treatment on various ecto-enzymes of cultured heart cells (fourth passage)*

BrdUrd (0.1 mM) was added 1 day after seeding and subsequently maintained in the medium, which was changed every second day. Enzyme analyses at pH 7.4 were performed on day 5 in culture, as described in the Experimental section.

Enzyme	Activity (nmol of P _i released/min per mg of protein)	
	Untreated	BrdUrd-treated
Ecto-(Ca ²⁺ -dependent ATPase)	39	220
Ecto-(Mg ²⁺ -dependent ATPase)	40	209
Ecto-(5'-nucleotidase)	70	53
Ecto-(phosphatase)	5	11

the effects of BrdUrd on the surface ATPase of serially passaged hamster heart cells were of a general kind. Assays were performed *in situ* after 4 days of treatment (i.e. on day 5 in culture), with untreated cultures serving as controls (Table 2). The basal activities varied considerably, but were stimulated by BrdUrd treatment in most cases. The most marked effects were obtained, however, when hamster fibroblasts or other hamster-derived cells were used.

The effects of BrdUrd treatment on other known ecto-enzymes were also studied. Ecto-(phosphatase) (*p*-nitrophenyl phosphatase; pH 7.4) (EC 3.1.3.1) was also increased, but less markedly, as a result of BrdUrd treatment (Bulmer *et al.*, 1976), whereas ecto-(5'-nucleotidase) activity was not altered by BrdUrd treatment of the cells (Table 3). This also shows that the measured ATPase activity was not

due to these enzymes, which in any case require Mg²⁺.

As noted previously (Coetzee *et al.*, 1977), BrdUrd exerted its effects on the Ca²⁺-dependent ATPase only if treatment was started while the cells were actively dividing. Thus Ca²⁺-dependent ATPase activity was unaffected when monolayered cells were treated. As shown in Fig. 3, the ATPase activity was proportional to the concentration of BrdUrd added early in the culture, up to about 0.1 mM. At higher concentrations, no further increases were observed.

To establish whether BrdUrd affected the ecto-(Ca²⁺-dependent ATPase) through a DNA-linked mechanism, BrdUrd treatment was combined with various other nucleoside analogues as well as with thymidine (Table 4). Addition of 1- β -D-arabino-furanosylcytosine (Cohen, 1967) did increase ecto-(Ca²⁺-dependent ATPase) activity, but the effect was very much less than that given by BrdUrd treatment. When BrdUrd was combined with this analogue, the activity failed to increase beyond this value and thus prevented the major effect brought about by BrdUrd alone. FdUrd (Heidelberger, 1965), which inhibits thymidylate synthetase and therefore prevents DNA synthesis by depletion of the thymidine pool, also had little effect on its own, but when it was added in combination with BrdUrd (0.1 mM), a very high ecto-(Ca²⁺-dependent ATPase) activity was obtained, equal to that given by treatment with 0.1 mM-BrdUrd alone. When FdUrd treatment was combined with one-tenth the amount of BrdUrd, a higher activity was obtained than with BrdUrd alone at this low concentration. Depletion of the thymidine pool as a result of FdUrd treatment presumably resulted in enhanced incorporation of BrdUrd into DNA, so that effects equal to those seen at 10-fold higher

Fig. 3. *Dependence of ecto-(Ca²⁺-dependent ATPase) activity on BrdUrd concentration*

Ecto-(Ca²⁺-dependent ATPase) activities were expressed as a function of total cellular protein and were analysed, as described in the Experimental section, on day 5 in culture; BrdUrd had been added on day 1.

Fig. 4. *Effects of various BrdUrd treatment times on replated heart cells in culture*

Treatment with 0.1 mM-BrdUrd was started 12 h after seeding, and all analyses were performed 96 h later. (a) Ecto-(Ca²⁺-dependent ATPase) activity; (b) incorporation of Br[³H]dUrd into the nuclei. Details of procedures are given in the Experimental section.

Table 4. *Effect of nucleoside analogues and thymidine on the ecto-(ATPase) activity of replated cultured heart cells*

Cells were cultured and assayed *in situ* as described in the Experimental section.

Treatment of cells on day 1 in culture	Ecto-(ATPase) on day 5 (units/mg of protein)
Untreated	35
BrdUrd (0.01 mM)	127
BrdUrd (0.1 mM)	220
1-β-D-Arabinofuranosylcytosine (10 μM)	85
1-β-D-Arabinofuranosylcytosine (10 μM)+BrdUrd (0.1 mM)	84
FdUrd (0.1 mM)	48
FdUrd (0.1 mM)+BrdUrd (0.01 mM)	200
FdUrd (0.1 mM)+BrdUrd (0.1 mM)	190
Thymidine (0.1 mM)	69
Thymidine (0.1 mM)+BrdUrd (0.1 mM)	87

BrdUrd concentrations were brought about (Chaganti *et al.*, 1974). The presence of equimolar thymidine concentrations blocked the effects of BrdUrd. All these results point to the necessity for analogue

incorporation into DNA for the increase in surface ATPase to be expressed.

More direct evidence for a relationship between DNA synthesis and ecto-(ATPase) was sought by the use of radioautography. The cells were treated with 0.1 mM-BrdUrd for increasing lengths of time, after which they were washed with medium not containing BrdUrd, and then cultured in the absence of BrdUrd. All analyses were done after the cells had been cultured for a constant period of 96 h after treatment with BrdUrd had been started. Simultaneously, a second set of cells was treated in the same way with Br[³H]-dUrd, also at a final concentration of 0.1 mM. These latter cells were fixed, stained and examined by radioautography (Fig. 4). The greatest effects on the ecto-(Ca²⁺-dependent ATPase) were observed when treatment was prolonged for more than 24 h. It was also with similar long treatment times that the largest relative increases in the average number of grains per nucleus was obtained. In Plate 1 the accumulation of grains in the nuclei can be seen, the cytoplasm displaying far fewer grains. The general morphology of the nuclei changed as the exposure time to BrdUrd

EXPLANATION OF PLATE I

Incorporation of Br[³H]dUrd into nuclei of cultured cells during different periods of exposure
Treatment with 0.1 mM-Br[³H]dUrd was started 12 h after seeding, and continued for 2 h (a), 12 h (b), 48 h (c) and 96 h (d). The bar indicates 5 μ m. Procedures are described in the Experimental section.

was increased; from being elongated and oval, they became larger and rounder. Mitotic figures, which were present in 5% of the cells in the first 40h in culture, were not seen at later stages. The number of grains per nucleus, however, continued to increase beyond the time when no further increases in cell number could be observed. Repair mechanisms possibly accelerated by the presence of BrdUrd in DNA chains, and/or replicative DNA synthesis without mitosis (G_2 block), were possible explanations for this phenomenon.

Discussion

The principal effect of BrdUrd observed on hamster heart cells grown in culture is a dramatic increase in the activity of a Ca^{2+} -dependent ATPase which is not filamentous myofibrillar myosin. This activity is particulate in nature, because it can be precipitated by centrifugation. The BrdUrd-affected Ca^{2+} -dependent ATPase activity is located on the plasma membrane, since it can be assayed at the external surface of intact heart cells *in situ*; this also indicates that the active site is exposed on the outer surface of the cells. The fact that this activity has kinetic properties different from those of myofibrillar myosin does not rule out the possibility that myosin-like proteins localized in plasma membranes are involved. Such proteins have been detected in many cell types where the characteristic sarcomeric organization found in muscle cells is absent (for review, see Pollard & Weihung, 1974). Gwynn *et al.* (1974) and Willingham *et al.* (1974), using immunofluorescence techniques, have described findings with various types of cells in culture which indicate that myosins are integral components of the cell surface. Garnett *et al.* (1976) found that an antibody raised against purified chicken gizzard (smooth-muscle) myosin partially inhibited the Ca^{2+} -dependent ATPase activity of mouse liver plasma-membrane preparations. Membrane-linked myosins may well have enzymic characteristics in relation to cation activation and susceptibility to thiol reagents which are very different from those of myofibrillar myosin.

Possible physiological roles for an ecto- $(Ca^{2+}$ -dependent ATPase) of mammalian cells, other than one related to non-muscle movement as referred to above, include Ca^{2+} transport across the plasma membrane (Katz & Blostein, 1975; Shami *et al.*, 1975), an, as yet, undefined energy-coupled biosynthetic activity on the outer surface of cells, metabolic coupling between cells (Stefanovic *et al.*, 1976) or the detection of extracellular ATP as a chemotactic agent (De Pierre & Karnovsky, 1974a,b). The ATPase could also be an abortive activity of an entity normally utilizing other substrates, or an artifact. We tested verapamil and ouabain to check whether the ATPase was related to entities known to be involved in cation-

transport processes; both were not inhibitory. The effect of BrdUrd described in the present study could provide a model for the study of the above-mentioned possibilities. Many cell types grown in culture contain this ecto-enzyme, but only some respond to BrdUrd treatment, in increasing the specific activity of the enzyme. This action of BrdUrd does not thus seem to be a universal cell-surface effect. Whether this is related to the specific physiological aspects of the different cell types is as yet unknown.

These studies, together with those reported in the preceding paper (Coetzee *et al.*, 1977), indicate that Ca^{2+} -dependent ATPase activities should not be used in studies of muscle cell differentiation as a direct assessment of the concentration of myofibrillar myosin, at least not without characterization of the molecules in terms of cation-dependence or correlations with gel-band densities.

The increase in ecto- $(Ca^{2+}$ -dependent ATPase) activity in replated hamster heart cells after treatment with BrdUrd in culture is proportional to the concentration of BrdUrd used. It appears that BrdUrd must be incorporated into cellular DNA, to be able to exert this specific effect. Our data even suggest that this conclusion applies to replicative mechanisms of BrdUrd incorporation into DNA, which is known not to be random (Strom & Dorfman, 1976). One could argue that a 'hit' at a certain locus in each cell is required to produce a quantum ecto- $(Ca^{2+}$ -dependent ATPase) enhancement in the surface membrane; increasing the concentration of the analogue would then improve the chances of such a 'hit' and thus the average activity of the culture would increase in proportion, until all cells had been affected. Alternatively, BrdUrd-substituted DNA may be subject to extensive repair activity, which could increase the likelihood of 'hits' even when mitotic activity has ceased. Genetic disruption caused by BrdUrd incorporation could produce a secondary membrane disorganization associated with increased assayable ecto- $(Ca^{2+}$ -dependent ATPase) activity. Since not all ecto-enzymes tested are affected, it remains unclear whether the ATPase has any special role in the membrane functions of BrdUrd-treated cells, or whether it has anything to do with the known effects of BrdUrd on differentiative behaviour in a variety of cell types.

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