Alkaline Phosphatase in Synchronized Human Cells

The marked alkaline phosphatase (AP) activity of certain established human cell lines[1] has been the subject of considerable interest[2-5]. In 2 human cell lines maintained in this laboratory, HeLa[6] and RA[7], AP activity has been studied in cell sonicates using *p*-nitrophenyl phosphate as the substrate and in histochemical preparations using α-naphthol phosphate as the substrate and blue RR as the coupler.

By a method previously described[8], a high degree of synchronous DNA synthesis and subsequent mitotic activity can be induced in HeLa cells in suspension culture. We have employed this method to study AP activity during these portions of the cell cycle.

HeLa cells were grown in suspension in 1 l screw-cap flasks containing 200 ml of medium. The flasks were placed on a rotary shake table which performed 100 excursions/min. Synchronization was induced in experimental cultures by including 5-aminouracil (5-AU) at a concentration of $3 \cdot 10^{-3} M$ in the medium for 24 h. After this period, both control and experimental cultures were centrifuged and the cells were resuspended in fresh medium. The time of 5-AU removal was referred to as time zero. DNA synthesis was followed by measuring the amount of tritiated thymidine incorporated into a trichloracetic acid-insoluble fraction in a 30 min pulse label. Cell number was determined with an electronic counter. The details of this method, including medium composition, etc., have been described elsewhere[8]. All AP experiments employed medium containing inactivated calf serum (30 min at 56 °C) and without phenol red. For measurement of AP activity, 2.0 ml of culture were removed, placed in an ice bath, and sonicated for 30 sec; 0.5 ml of sonicate was added to the substrate mixture consisting of $0.016 M$ disodium *p*-nitrophenyl phosphate plus $1 M$ 2-amino-2-methyl-1-propanol-HCl at pH 10.6[9]. After 20 min of incubation at 37 °C, 2 ml of $0.25 N$ NaOH were added to stop the reaction. The amount of *p*-nitrophenol liberated was measured at 410 nm using a Bausch and Lomb Spectronic 20 photoelectric colorimeter. Varying sonicating time from 15 sec to 4 min and/or lysing cells with 1% desoxycholate did not significantly alter AP activity in control or synchronized cells.

Figure 1A shows DNA synthesis in control and 5-AU-treated cells. Immediately after removal of the analog there was a burst of DNA synthesis which peaked at about 3–4 h, then dropped below control level at about 8 h and remained minimal during the rest of the experiment. Figure 1B shows the number of cells per ml in both cultures. Control and experimental cell numbers increased at the same gradual rate between 1 and 9 h. However, from 9–12 h there was a marked increase in cell division in the 5-AU-treated cells. From these data it would appear that in the 5-AU-treated culture a large number of cells were synthesizing DNA synchronously and, subsequently, dividing synchronously. AP activity, however, shown in Figure 1C, remained at essentially the same level throughout in both control and treated cells. Figure 1B shows that the cell number in the 5-AU-treated cells increased by a factor of about 1.5 during the interval from 9–12 h. In other words, about half the cells divided during this interval. Mitosis in these cells requires about $^3/_4$ h for completion. Therefore about 10% of the cells must have been in mitosis at any one moment. In this same period, there could have been very few mitoses in the controls. Figure 1C shows that even this difference in the number of mitotic cells had no effect on the amount of alkaline phosphatase detected in the culture. It can be calculated that a four-fold increase in the AP content of mitotic cells could certainly be detected by this method and quite probably a three-fold one.

Although from 0–9 h the number of cells in both control and experimental cultures was essentially the same, the AP activity in the 5-AU-treated cells appeared consistently somewhat higher. In a number of experiments.

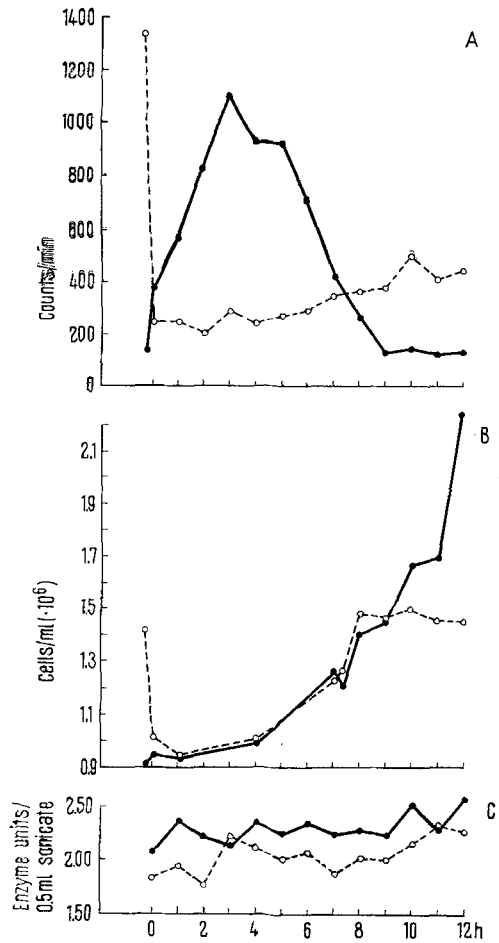

Fig. 1. (A) DNA synthesis. (B) Cell number. (C) AP activity in control and 5-AU-treated cells. 1 enzyme unit = AP activity in 0.5 ml of sonicate sufficient to hydrolyze 1 mM/ml of *p*-nitrophenol in 20 min at 37 °C. o—o control. ●—● 5-AU-treated.

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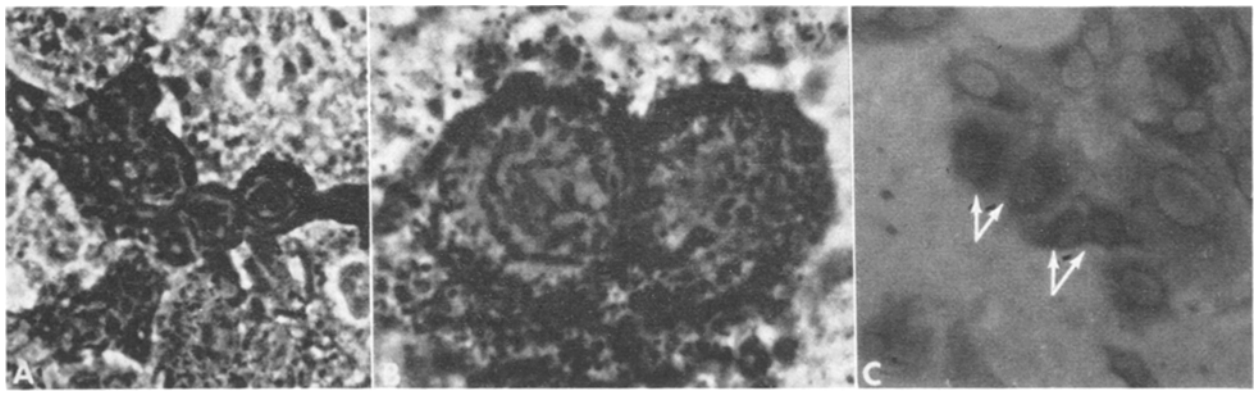

Fig. 2. Mitotic and post-mitotic cells stained for alkaline phosphatase. (A) Darkly-staining dividing cells. (B) Enlargement of dividing cells. (C) Darkly-staining post-mitotic cells (arrows).

5-AU-treated cells have always appeared to have a slightly larger volume than control cells; if cells are left for 60 h in 5-AU, giant cells up to 20 times normal size result[10]. Thus, one would expect 5-AU-treated cells to possess greater amounts of such cytoplasmic constituents as AP. 5-AU synchronization seems to induce an extended G_1. Effects of this on cell metabolism not detected by the methods used here cannot, of course, be excluded.

In cells assayed histochemically for AP with α-naphthol phosphate (Figure 2), dividing cells appeared to have an increased AP activity (Figure 2, A and B). However, the data presented above seem to indicate that AP activity does not increase at division. Therefore it is possible that the apparent increase seen histochemically in dividing cells is merely an effect produced by the rounding-up of the cells at division. More difficult to explain, however, is the apparent increased AP activity around the nuclear membrane in post-mitotic cells (Figure 2C).

Since it was possible that the 2 different substrates involved were assaying different entities, experiments were performed utilizing α-naphthol phosphate quantitatively.

Control and 5-AU-treated static HeLa cultures were harvested 11 h after 5-AU removal by scraping the cells from the culture surface with a rubber policeman. At this time the mitotic index of the control cells was 1.3%; that of the experimental cells, 10.8%. The cells were centrifuged, resuspended in Hanks' balanced salt solution (without phenol red), and cell number was adjusted to 10^6 cells/ml in both suspensions. The cells were sonicated, and 0.5 ml of sonicate was added to 2 ml of α-naphthol phosphate-blue RR substrate mixture[11]. After 20 min of incubation at 37 °C, 3 ml of concentrated HCl were added and optical density at 600 nm was determined (Table). Preliminary experiments with this method using hog mucosa AP (Sigma) showed that the optical density developed was dependent on enzyme concentration.

The results with α-naphthol phosphate used in this fashion also indicate that there is not an increased AP activity in dividing and post-mitotic cells. Thus the apparent increased activity seen histochemically in dividing cells would seem to be an effect produced by the rounding-up of mitotic cells. The high activity frequently seen around the nuclear membrane of post-mitotic cells may well reflect subcellular movement of AP to this site at this time, rather than an increase in total AP. This provides some basis for speculation as to the role of AP, the function(s) of which are unknown[2,12].

AP activity with α-naphthol phosphate as substrate

Sample (0.5 ml)	Optical density 600 nm
Hanks' BSS	0.240
Medium	0.201
Control cell sonicate	1.005
5-AU-treated cell sonicate	0.995

Zusammenfassung. Die Aktivität der alkalischen Phosphatase (AP) wurde in mit 5-Aminouracil synchronisierten Suspensionskulturen menschlicher Zellen histochemisch unter Benutzung von α-Naphtholphosphat als Substrat und chemisch unter Benutzung von *p*-Nitrophenylphosphat als Substrat untersucht. Die histochemischen Untersuchungen weisen auf eine gesteigerte AP-Aktivität in mitotischen und postmitotischen Zellen, insbesondere im Bereich der Kernmembran hin. Im Gegensatz dazu zeigen die Experimente mit synchronisierten Zellen eine weitgehend konstante AP-Aktivität während der gesamten DNS-Synthese, Mitose und unmittelbaren postmitotischen Phase. Die Ergebnisse der histochemischen Untersuchungen sprechen daher eher für eine intrazelluläre Verlagerung des Enzyms mit spezifischer Lokalisation und nicht für eine Steigerung der AP-Aktivität.

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[13] USPHS Postdoctoral Fellow (National Cancer Institute Fellowship No. 2-F2-CA-13, 666-02).