CELL CYCLE EVENTS IN THE HYDROCORTISONE REGULATION OF ALKALINE PHOSPHATASE IN HELA S_3 CELLS

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ABSTRACT

The increase in alkaline phosphatase in asynchronous cultures of HeLa S_3 cells grown in medium supplemented with hydrocortisone is characterized by a lag period of 10–12 hr. Present studies utilizing synchronous cell populations indicate: (*a*) a minimum of 8–10 hr of incubation with hydrocortisone is necessary for maximum induction of alkaline phosphatase; (*b*) the increase in enzyme activity produced by hydrocortisone is initiated exclusively in the synthetic phase of the cell cycle; (*c*) alkaline phosphatase activity does not vary appreciably over a normal control cell cycle. Radioactive hydrocortisone is rapidly distributed into HeLa cells irrespective of their position in the cell cycle, indicating that inductive effects are not governed by selective permeability during the cell cycle. Hydrocortisone-1,2-[^{3}H] diffuses back from the cell into the medium when the cells are incubated in fresh medium containing no hydrocortisone, and the alkaline phosphatase induction, under these conditions, is completely reversible.

INTRODUCTION

The alkaline phosphatase activity of HeLa S_2 cells has been shown to increase when the cells are grown in medium containing the adrenal glucocorticoid hormone hydrocortisone (1–6). This induction of alkaline phosphatase in HeLa S_3 cells is characterized by an extended lag period which occurs after addition of the steroid hormone (1, 2). This type of induction is in contrast to hydrocortisone-mediated induction of tyrosine-α-ketoglutarate transaminase in a rat liver cell line (7) which begins after 1 hr and reaches a peak at 6 hr and is also in contrast to prednisolone-mediated induction of alkaline phosphatase in human leukocytes in vivo (8) which begins after 3 hr.

The aims of the present study were to use synchronous cell cultures to define age-dependent responses of HeLa S_3 cells to hydrocortisone in eliciting the induction of alkaline phosphatase, and to use radioactive hydrocortisone to determine how cell permeability and cellular concentration of hydrocortisone relate to the induction process.

MATERIALS AND METHODS

Cells and Media

The HeLa S_3 cell line used was originally cloned for continuous suspension culture, but has been adapted to monolayer culture (2). These cells were grown in monolayer cultures, at 37°C, in Eagle's Minimum Essential Medium (MEM) supplemented with 9% calf serum as described elsewhere (1, 2). Induction kinetics were usually studied in plastic T_{25} flasks (Falcon Plastics Co., Los Angeles), but the results obtained in glass bottles were comparable. The majority of the radioactive studies were per-

formed on cultures in glass milk dilution bottles containing 2.5 times as much medium and cells (10 ml) as the plastic T_{25} flasks (4 ml).

Assay Procedures

Total alkaline phosphatase activity was obtained from a sodium deoxycholate lysate (9). The enzyme activity of this lysate was determined spectrophotometrically at 410 mμ in the following manner: 0.1 ml of lysate was added to 0.4 ml of *p*-nitrophenyl phosphate (8 mM) in 0.25 M Tris buffer, pH 10.0, containing $MgCl_2$ (1 mM), and the mixture was incubated at 37°C until a yellow color appeared, whereupon the reaction was stopped with 1 ml of 0.25 M NaOH. The enzyme assays were performed in triplicate on freshly thawed samples and generally gave a standard error of less than 3%. All lysates of samples were rapidly frozen in Dry Ice–acetone (−80°C) at each individual time point unless otherwise specified. The average loss in activity in freshly thawed samples was uniform and was about 5% of the total activity. Protein was assayed by the method of Lowry et al. (10).

Radioactive Isotope Incorporation Studies

In certain experiments cells were grown in medium containing hydrocortisone-1,2-[^{3}H] (2 μg/ml; specific activity, 730 mc/mmole) supplied by New England Nuclear Corporation, Boston. After gentle scraping, the harvested cells were washed thrice by repeated centrifugation in isotonic saline containing 1 mM Tris pH 7.5. The first wash contained 1% of the total initial counts of the medium; the second wash contained 5% of the counts of the first wash, and the third wash contained 5–10% of the counts of the second wash, which was less than the total radioactive counts of the corresponding cellular fraction. Thus the counts of the lysate represent cellular-associated material and not extracellular-medium-associated material.

Samples were counted in Bray's dioxane scintillation solution (11) in a Nuclear-Chicago liquid scintillation counter (Mark I, Nuclear-Chicago Corporation, Des Plaines, Ill.) and all values were corrected to disintegrations per minute (dpm) by use of an automatic external standard and an acetone quench curve.

Back Exchange Experiments

HeLa S_3 cells were grown in MEM supplemented with hydrocortisone-1,2-[^{3}H] (2 μg/ml) for various lengths of time, after which the cells were washed with MEM and grown for the remaining time in medium which contained no hydrocortisone for a total of 75 hr. Thus the kinetic aspect of these experiments is the length of time cells were allowed to grow in the presence of hydrocortisone-1,2-[^{3}H] compared to the total length of time of growth (75 hr). The period in which the cells, preincubated with hydrocortisone-1,2-[^{3}H], were grown in medium containing no hydrocortisone is defined as the back exchange period (12). Had we transferred the cells to a medium containing nonradioactive hydrocortisone, this period of incubation with the steroid would have been defined as a chase period. During the back exchange period there is an infinitely dilute gradient against the intracellular hydrocortisone-1,2-[^{3}H]. This type of experiment was compared to a direct induction performed at the same time. In order to give a valid comparison of alkaline phosphatase activity, the direct induction samples were frozen rapidly (−80°C) and stored at −20°C from the time of collection until 1 day after termination of the back exchange samples. The back exchange samples, all collected at 75 hr, were frozen rapidly (−80°C) and cell samples were analyzed for phosphatase activity after one thawing.

Synchronous Cell Selection

Cells were grown in low-calcium Eagle's MEM supplemented with 9% calf serum (13, 14). The only calcium in the complete growth medium is that supplied by the calf serum. After 20–24 hr the bottles were gently shaken by hand for 10–15 sec in calcium-supplemented MEM and the cells were explanted into monolayer culture. The cells that were dislodged represent a synchronous population of predominantly mitotic cells. A small aliquot of these cells were fixed in methanol-acetic acid and stained with Giemsa's. Light microscopy indicated that 80–85% of these cells were in mitosis. An indication that partial synchrony was maintained for at least one cell cycle was provided in several experiments by total cell counts. After a small initial decrease in cell numbers, the cell count remained practically constant for the first 18 hr. An increase of about 75–80% in cell numbers was observed in several experiments between 18–20 hr after selection, indicating a maintenance of synchrony within this cell population.

RESULTS

Induction of Synchronous and Asychronous Cells

The average doubling time of logarithmically growing asynchronous and synchronous HeLa S_3 cells under the conditions employed for monolayer culture was 18.5 ± 1.5 hr. The size of the initial inoculum of cells was a critical factor in establishing a maximally doubling population. An inoculum of less than 10^5 cells/ml complete medium tended to increase the doubling time to as long as 30 hr.

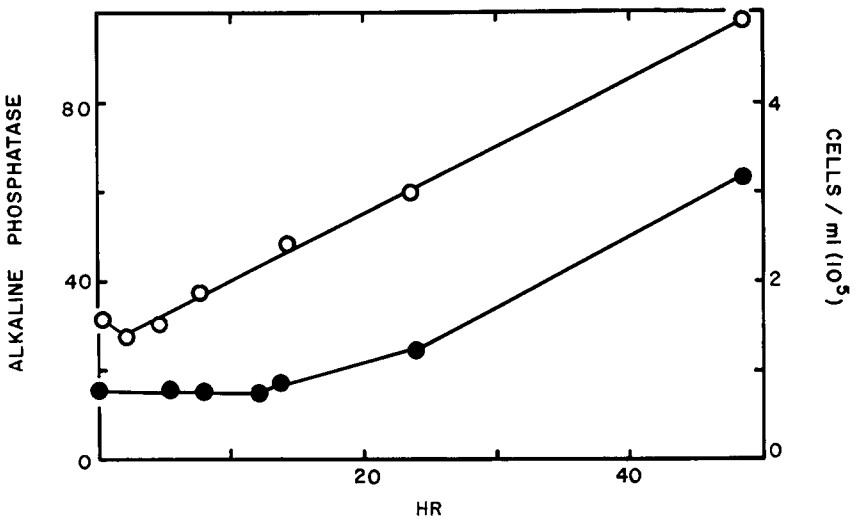

FIGURE 1 Hydrocortisone induction of alkaline phosphatase in asynchronous HeLa S_3 cells. The specific activity of alkaline phosphatase is expressed as μmoles of pNPP (*p*-nitrophenyl phosphate) hydrolyzed per min per mg protein. ●—● Alkaline phosphatase (specific activity); ○—○, cells per ml.

Freshly trypsinized asynchronous cells were allowed to grow for at least 16 hr before addition of hydrocortisone, in order to correct for lag and plating loss produced by the explanting procedure. As seen in Fig. 1, the induction of alkaline phosphatase in this HeLa cell clone is characterized by a lag period of about 12 hr. The cell kinetics indicate an asynchronous, exponentially distributed cell population. Fig. 2 illustrates induction kinetics of alkaline phosphatase in synchronous cell populations; hydrocortisone was added at various time periods after mitotic cell selection, and the figure is a composite of three experiments. Several other experiments gave similar results. The induction of this enzyme occurs in one portion of the cell cycle. Synchronous cultures which received hydrocortisone 12 hr after selection evidenced no significant enzyme increase until shortly after 32 hr, which is the time for one complete cycle (+20 hr) and one 12-hr period. Even more pertinent, when hydrocortisone was added 25 hr after selection, the induction curve was superimposable with that of the +12 hr set. Some induction is evident when hydrocortisone is added 30 hr after selection (□—□, Fig. 2), but the rate of increase from 30 to 50 hr is less than the rate during the same period of the +25 hr set. Fig. 2 also gives the cell number versus time, and suggests that a reasonable amount of synchrony was maintained after this length of time in culture. The cell number for treated and control experiments was very similar through the 37th hr (second expected mitosis), but a sharp increase in cell number at this point was obliterated in both treated and control populations because of monolayer packing and concomitant contact inhibition (at about 3×10^5 cells/ml) and, presumably, desynchronization of the populations after the first mitosis. Also illustrated in Fig. 2 (-○-○-) is a series of experiments in which hydrocortisone was added immediately after selecting mitotic cells (zero time). These experiments showed an increase in alkaline phosphatase activity that was somewhat less than that observed at +32 hr, but the results in Fig. 2 (-○-○) again show that the point of initiation of induction occurs 12 hr after mitosis. The inefficient response of freshly selected cells was not investigated further since the physiology of cells transplanted from low-calcium medium to normal-calcium medium is poorly understood. Calcium in high levels is not necessary for induction, since HeLa S_3 cells grown with hydrocortisone in low-calcium MEM show a four- to sixfold induction in alkaline phosphatase activity compared with their controls, and control alkaline phosphatase activity is comparable to the alkaline phosphatase activity of cells grown in normal MEM.

The steady level of alkaline phosphatase activity in synchronous cell populations to which no hydrocortisone was added is illustrated in Fig. 3. Several

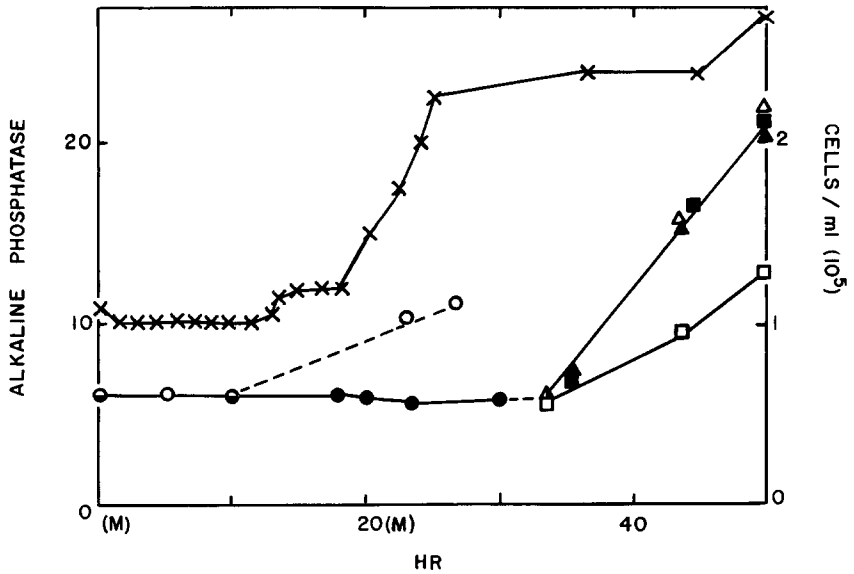

FIGURE 2 Hydrocortisone induction of alkaline phosphatase in synchronous HeLa S_3 cells. ●—●, Alkaline phosphatase (specific activity) in untreated cells; ○—○, hydrocortisone added at selection (zero time); △—△, hydrocortisone added at + 21 hours; ▲—▲, hydrocortisone added at + 25 hours; □—□, hydrocortisone added at +30 hours; ■—■, hydrocortisone added at + 12 hours. ×—× cells per ml. Midmitosis is indicated by (*M*).

additional experiments gave essentially similar results. The enzyme has only a mild fluctuation over the cell cycle, and, in view of the statistical spread obtained from pooling three separate experiments, could be best described as invariant over the entire cell cycle.

Cellular Content of Hydrocortisone-1,2-[^3H]

Since cell permeability during the cell cycle could be an explanation for the observed induction behavior of synchronous cells, an experiment in which tritiated hydrocortisone was added to synchronous cells was performed. The behavior of the alkaline phosphatase of these cells is illustrated in Fig. 2 (-○-○-). The radioactivity measure in thrice-washed, whole cell lysates was generally invariant over the cell cycle, being 1.4×10^5 dpm/10^6 cells at zero time, 1.9×10^5 dpm/10^6 cells at +4 hr, 1.5×10^5 dpm/10^6 cells at +12 hr, and 1.4×10^5 dpm/10^6 cells at +22 hr. The total incorporation of hydrocortisone-1,2-[^{3}H] in HeLa S_3 cells after as much as 72 hr is low, being 0.1 to 0.2% of the amount added. This is the amount expected from the ratio of cellular volume (0.011 ± 0.002 ml/5 × 10^6 cells)[1] to the total volume of medium (10.0 ml), indicating that the cells neither absorb nor exclude this steroid.

The continuous presence of hydrocortisone is necessary for continuous alkaline phosphatase induction. Table I presents, for comparison, the phosphatase activity during a continuous induction and both the alkaline phosphatase activity and the radioactivity of cells during a comparable back exchange experiment. After 3 hr of back exchange (72-hr point, Table I), the alkaline phosphatase level was similar to that at the 75-hr direct induction time point. However, after 27 hr of back exchange (48-hr point, Table I), the alkaline phosphatase activity was considerably less than that at the 75-hr direct induction point, although the amount of activity was the same as that at the 48-hr direct induction point (the cells being harvested at 48 hr).

The ready back diffusion of radioactive hydrocortisone parallels the decrease in induction of alkaline phosphatase. The amount of hydrocorti-

[1] This value is an average of three separate experiments measuring cell volume in a McNaught and MacKay-Shevky-Stafford sedimentation tube.

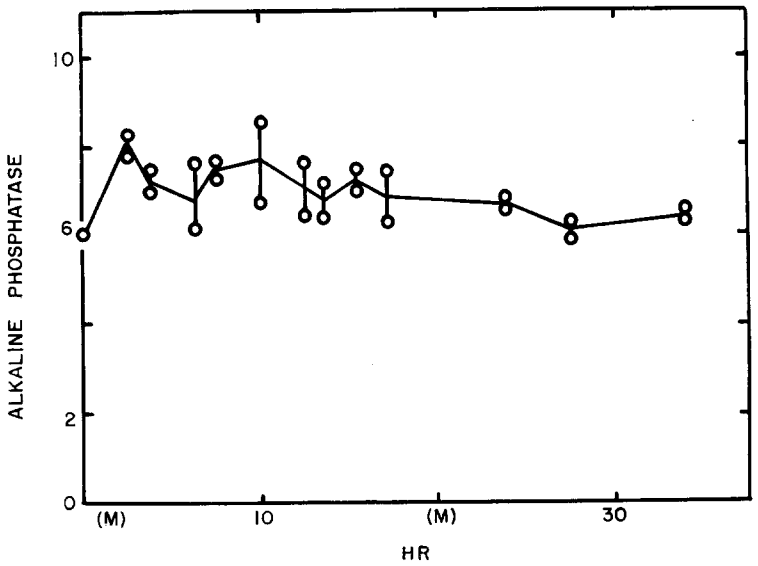

FIGURE 3 Variation in the specific activity of alkaline phosphatase in synchronous HeLa S_3 cells over a cell cycle. Midmitosis is indicated by (*M*). This figure represents a pooling of data of three separate experiments. Vertical spread indicates standard deviation from the mean.

sone remaining in cells and medium during back exchange (considering the steroid specific activity) is 0.002 μg of hydrocortisone/ml medium, which is less than the minimal dose necessary for induction (Table II). The irreversibly absorbed radioactivity (Table I, time points 0, 3, 10.5, and 24 hr with 1400 $\pm$ 500 dpm) is not physiologically important in alkaline phosphatase induction.

DISCUSSION

The population doubling time (15) HeLa S_3 cells in monolayer culture at 37°C is estimated by us to be 18.5 $\pm$ 1.5 hr, which is close to the determination of Rao and Engelberg (16) who found a mean population doubling time of 21.8 hr for HeLa S_3 cells in suspension culture at 37°C. The selection method which we used in this work for obtaining synchronous cultures is considered to be one of the most satsifactory methods of obtaining synchronous cell populations with maintenance of maximum physiological integrity (17), although the yield is modest and the synchrony is gradually lost. This method of selection results in a fair yield of relatively pure mitotic cells, and almost all the cells enter the proliferative pool as evidenced by the increase in cell numbers obtained from cultures 18–20 hr after selection (13, 14). The kinetics of induction of alkaline phosphatase in synchronous populations of HeLa S_3 cells indicate that the effect of hydrocortisone is to potentiate some event(s), 12 hr after mitosis, that results in the production of an alkaline phosphatase protein with increased phosphatase catalytic capability (3). At 12 hr after mitosis HeLa S_3 cells are in the synthetic (S) phase of the cell cycle (18). Although the initiation of induction can be demonstrated after the first 12 hr after synchronous selection, it is most effectively elaborated after one round of division plus 12 hr (about + 32 hr, Fig. 2). The behavior of asynchronous cell populations can be interpreted as a progression of cells through S, in which induction is elicited, *plus* about a 10–12 hr lag period (Fig. 1). Presumably, cells continue to progress through S during the first 11 hr after the addition of hydrocortisone to the asynchronous cells, but induction of enzyme occurs after an 11-hr lag period. Thus the cell age-dependent response to hydrocortisone is a necessary but not sufficient requirement for efficient induction. The poor response of synchronous cells to hydrocortisone when added +30 hr after selection compared to the response when hydrocortisone is added +25 hr after selection indicates that some adaptive process requiring cell-hydrocortisone interaction for at least 7 hr before middle S phase is also necessary for induction. This is in general agreement

TABLE I

*Alkaline Phosphatase Activity and Radioactivity of HeLa S_3 Cells Grown with Hydrocortisone-1,2-[^3H]**

Time‡	Back exchange experiment: Alkaline phosphatase specific activtiy	Back exchange experiment: dpm/10^6 cells	Direct induction: Alkaline phosphatase specific activity
Hr			
0	0.23	1,700	0.25
3	0.29	1,000	—
10.5	0.28	1,600	—
24	0.35	1,700	0.25
48	0.77	2,700	0.72
72	1.17	21,000	1.05

* Each bottle received 1.1 × 10^8 dpm hydrocortisone-1,2-[^{3}H].

‡ Time for back exchange samples indicates when medium was exchanged; for example, 24 hr indicates that the culture grew for 24 hr with radioactive steroid and then grew for 51 hr (75 minus 24) in normal MEM. All of the cells were sacrificed at 75 hr, washed three times in buffered isotonic saline, lysed in deoxycholate, and assayed as described in Materials and Methods.

TABLE II

Variation in Alkaline Phosphatase Activity With Concentration of Added Hydrocortisone

Hydrocortisone	Hydrocortisone	Alkaline phosphatase specific activity*
μg/ml	*nmolar*	
0	0	0.325
0.007	20	0.452
0.015	41	0.578
0.030	83	0.782
0.075	210	1.140
0.150	410	1.195
0.300	830	1.285
0.750	2070	1.291
1.500	4130	1.402
3.000	8260	1.330

* All cells grown, with the concentration of hydrocortisone listed, for a total of 72 hr.

with the lag period of about 11 hr for asynchronous cells discussed above.

The initiation of alkaline phosphatase induction at 12 hr after mitosis occurs after the cell has been in the S phase for about 4 hr, since the G_1 portion of the HeLa S_3 cell cycle is about 8 hr (18). Recently, Pfeiffer and Tolmach (19) have shown that shortly after the HeLa S_3 cell enters S a doubling in the rate of RNA synthesis occurs. Since the induction of alkaline phosphatase in HeLa S_3 cells has been shown to require new messenger RNA synthesis (2), it is possible that the messenger RNA for this new alkaline phosphatase protein is synthesized predominately in early S, and that 3–4 hr are required for messenger RNA processing and assemblage into a functioning cytoplasmic polysome before new RNA-directed enzyme protein synthesis begins. The linear rate of enzyme increase for at least one cell cycle after initiation suggests that the alkaline phosphatase protein is synthesized uniformly over the cell cycle. Confirmation of the rate of alkaline phosphatase synthesis at different times in the cell cycle awaits analysis of synchronous cell populations by protein pulse-labeling and characterization of the alkaline phosphatase-antibody complex to estimate the rate of synthesis of new enzyme protein as previously described (3).

The steady-state level of alkaline phosphatase is relatively constant in control HeLa S_3 cells during the cell cycle. This observation is completely compatible with the behavior of alkaline phosphatase in the acute phase of its induction by hydrocortisone which is initiated during the S phase of the cell cycle. After 70 hr of growth with hydrocortisone, HeLa S_3 cells have a new steady-state level of alkaline phosphatase which is 5–10 times greater than control levels (2). Since steady-state levels of enzymes are a resultant of both synthesis and degradation of the enzyme protein (turnover), and since messenger RNA for alkaline phosphatase is relatively long-lived (12 hr, reference 2), no great variation in total enzyme activity in the control or induced steady-state during the cell cycle is a necessary result of initiating the acute induction during S phase. The uniformity of alkaline phosphatase over the cell cycle in control HeLa S_3 cells is consistent with the findings of Regan (20). In all probability, the clone of HeLa cells which we have utilized is different from the clone that Regan used, since the control levels of the enzyme from these two clones are widely different. In another study (21) two human heteroploid cell clones were shown to regulate their alkaline phosphatase activity in response to hydrocortisone shortly after mitosis in early G_1. It is possible that differences in methods of obtaining synchronous

cells or differences in clone type account for the disparity between that study and this one.

Experiments with radioactive hydrocortisone and synchronous cells indicated that the cells had no particular permeability barrier to hydrocortisone during any point in the cell cycle. When cells were allowed to grow in the presence of hydrocortisone-1,2-[^{3}H] for varying lengths of time and then changed to medium without hydrocortisone, it became evident that continued incubation of the cells in medium containing hydrocortisone was necessary to achieve maximum induction of alkaline phosphatase. The amount of hydrocortisone-1,2-[^{3}H] remaining in the back exchange medium was in itself incapable of inducing alkaline phosphatase (see Table II). The ready reversibility of the cortisol induction of alkaline phosphatase illustrates that the interaction between steroid and HeLa cell is different from that between colchicine and cultured human KB cells (22). In a study of the kinetics of inhibition and the binding of colchicine (22), radioactive colchicine was used in a type of back exchange experiment. The data from that experiment together with a dose-response curve indicated that, as long as the bound colchicine per cell exceeded a critical value, the majority of the cells were blocked in metaphase after 6–8 hr of exposure to this spindle-inhibiting agent. Even when HeLa cells which have been treated with hydrocortisone for 48 hr are reincubated in control medium (back exchanged), alkaline phosphatase induction ceases and eventually the cells acquire control levels of this enzyme (2).

In another report (23), hydrocortisone was shown to be necessary before the completion of mitosis in proliferating cells of mouse mammary gland in order to allow prolactin to induce a differentiated cell type which synthesizes casein. In the present study, the inductive process was shown to be a property of S phase cells, although a minimum of 7 hr growth with cortisol was needed before a cell population began to show the induction of alkaline phosphatase.

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