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ACTIVITY OF GLYCOGEN METABOLIZING ENZYMES IN GLUCOSE DEPRIVED HT 29 ADENOCARCINOMA CELL-LINE

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When deprived of glucose, the cultured HT 29 adenocarcinoma cells are able to mobilize their glycogen within 4 hours. Glycogen phosphorylase is strongly activated during the first hour of glucose starvation. Then, while the a/a+b ratio for phosphorylase is declining, glycogen synthase is partially converted into the a form ; this conversion does occur although glycogen phosphorylase is far from being totally inactivated. After 4 hours, activity of both a and total forms of glycogen synthase decrease.

Cell UDP-glucose and glucose-6-P levels are declining during the 24 hours period of glucose starvation. Cell ATP content decreases by only 50 percent over the same period of time.

The biological changes observed during carcinogenesis are manifold and also concern glycogen metabolism. This is well established in the case of hepatocarcinoma (1). It has also been recently shown that human adenocarcinoma store high levels of glycogen as compared to the normal corresponding epitheliums (2,3,4). When established in permanent culture, these cancer cells accumulate glycogen in a growth related way : during the exponential phase, glycogen stays at a basal level which is characteristic for each line and roughly identical to what is found in the corresponding tumor ; after confluence, when the rate of cell division slows down, the glycogen content increases to reach considerable values (2,3). Moreover, studies on synchronized cells in culture have shown that the pattern of glycogen storage was linked to the cell cycle, with a peak during the G_1 phase (2).

In normal mammalian tissues, glycogen metabolism is controlled by changes in glycogen synthase and phosphorylase activities due to changes in the phosphorylation state of these enzymes. Previous results on the control of glycogen deposition during the culture of several adenocarcinoma cell lines indicated that the glycogen synthase is never found in the active a form (a/a+b ratio stays under 0.10 at any time of the culture) and that glycogen synthesis may be achieved by glycogen synthase b allosterically activated by the high concentration of glucose-6-P (5). One of these cell lines (HT 29) has

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also been shown to possess an adenylate cyclase system very sensitive to vasoactive intestinal peptide (VIP) as this hormone provokes a large increase of cyclic AMP level and the successive activation of cyclic AMP dependent protein kinase and glycogen phosphorylase, followed by a moderate glycogenolysis (6, 7,8).

These results have led us to study the glycogen metabolism in the HT 29 cell line in the experimental case of a rapid glycogen breakdown induced by glucose deprivation.

MATERIALS AND METHODS

Cells were cultured in 25 sq.cm. plastic flasks from Corning Glass Works (Corning, USA). Dulbecco's modified Eagle medium from Eurobio (Paris, France) was supplemented with 10 percent fetal calf serum from Gibco (New-York, USA).

The following chemicals were purchased from the indicated sources : UDP-glucose, glucose-6-P, glucose-1-P, 5'AMP, NAD, NADP, UDP-glucose dehydrogenase and glucose-6-P dehydrogenase from Boehringer Mannheim (Mannheim, Germany) [^{14}C] glucose-1-P and UDP - [^{14}C] glucose from New England Nuclear Corp. (Boston, USA) ; oyster glycogen type II and all other compounds from Sigma Chemical Company (St Louis, USA).

The bioluminescence ATP monitoring kit was a generous gift from the LKB-Wallac Company.

Cell culture and experimental procedure. The HT 29 cell line has been established in permanent culture by Dr. J. FOGH, from a human colon adenocarcinoma (9). The cells are grown in Dulbecco's modified Eagle medium containing 25 mM glucose and supplemented with 10 percent fetal calf serum.

Our experiments are carried out with cells in stationary phase, 12 hours after the last medium change. The experimental period starts when the glucose containing medium is removed and replaced by a glucose free medium. After different times, the medium is sucked out, and the cell layers are rapidly washed with ice-cold saline, then immediately frozen and stored at -80°C until analysis. Three flasks, run in parallel, are used at each step of the experiment.

Glycogen and protein determination. Glycogen is measured according to Van Handel (10) using anthrone. Protein content is determined by the method of Bradford (11).

Glycogen synthase and phosphorylase assays. For enzyme activities measurements, the frozen cell layers are scraped in an ice-cold buffer containing : 40 mM imidazole (pH 7.2),8 mM EDTA, 100 mM NaF and 40 mM mercaptoethanol. The cells are disrupted by short sonication and the homogenate centrifuged (2 min, 4°C, 9 000 g). Glycogen synthase and phosphorylase are assayed in supernatant as described by Sato et al. (12) and Wang et al. (13) respectively. Activities are expressed as nmoles of UDP-Glucose or Glucose-1-P incorporated into glycogen/min/mg of protein at 30°C.

Measurement of ATP. ATP extraction was performed as described in (14) : Cell layers are treated with 2 ml of a 10 percent trichloracetic acid solution containing 4 mM EDTA; after 5 min, aliquots of the extract are centrifuged (2 min, 4°C, 9 000 g), diluted 1 000 folds and processed from ATP measurements by the luciferin-luciferase method using an ATP monotoring kit from LKB-Wallac and a LKB 1251 luminometer.

Measurement of Glucose-6-P and UDP-Glucose. Glucose-6-P and UDP-Glucose concentrations are measured by adapting the enzymatic methods of Lang et al. (15) and Keppler et al. (16), respectively. Assays are processed after deproteinization of the cell homogenates with 3.5 percent perchloric acid followed by neutralization with a pretitrated volume of 4 M $KHCO_3$.

RESULTS

Effect of glucose deprivation on glycogen content

HT 29 cells grown in presence of 25 mM glucose and taken 12 hours after the last medium change are found to contain 44.3 ± 2.6 (S.D.) µg of glycogen per mg of cell protein. When the cells are starved for glucose, their glycogen content falls sharply : 75 percent of the glycogen stores disappear after 2 hours and only trace amounts of the polymer (3.4 ± 0.4 (S.D.) µg of glycogen per mg of cell protein) are found after 4 hours (Fig. 1). When prolonging glucose deprivation for longer periods up to 24 hours, the cell glycogen content remains low.

Effect of glucose starvation on glycogen phosphorylase activity

The results concerning glycogen phosphorylase activity are shown in Fig. 2. When assayed in presence of 1 mM AMP, total phosphorylase activity is about 320 nanomoles of glucose-1-P incorporated into glycogen/min/mg of protein and remains constant over the whole experiment period.

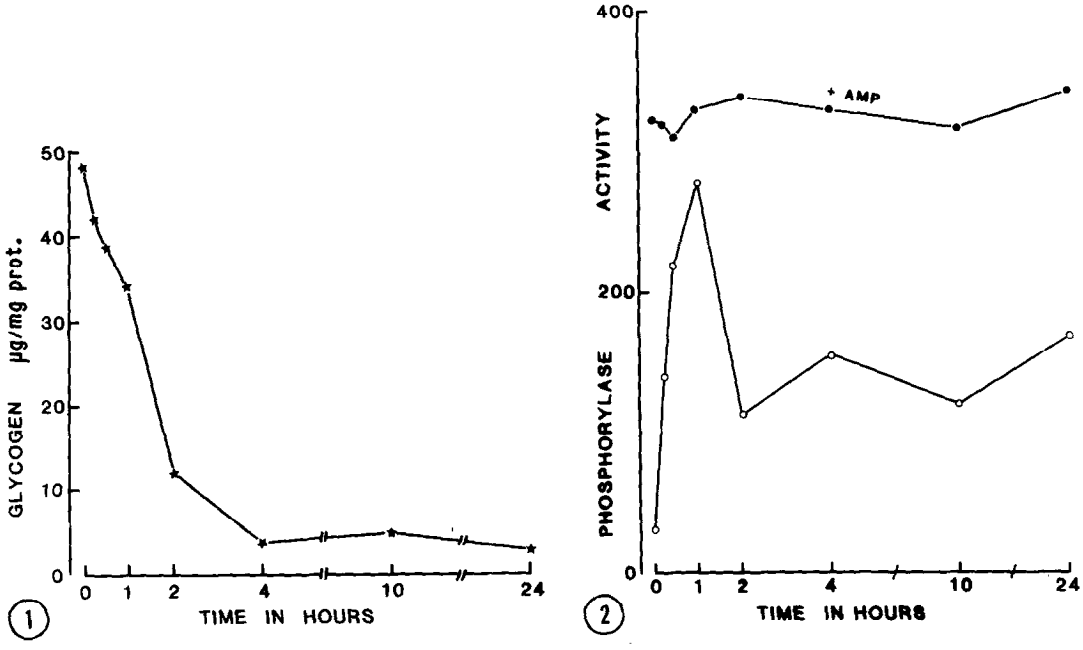
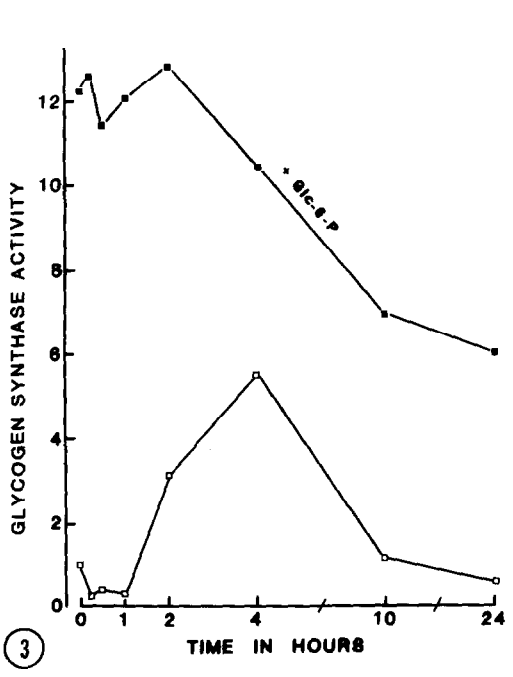


Fig 1. Effect of glucose starvation on HT 29 cell glycogen content. HT 29 cells were grown to post confluent stage in Dulbecco's modified Eagle medium supplemented with 10 percent fetal calf serum and containing 25 mM glucose. At zero time, the medium was removed and changed by glucose free medium. At indicated times, cell monolayers were taken, washed with ice-cold saline solution and frozen until analysis. Glycogen was measured using the anthrone method and the concentrations are expressed as µg/mg of cell protein.

Fig. 2. Effect of glucose starvation on glycogen phosphorylase activity. Experimental conditions were the same as in Fig. 1. Glycogen phosphorylase activity was measured without (○) or with (●) 1 mM AMP, using the method described by Sato et al. (12). Activities are expressed as nanomoles of glucose-1-P incorporated into glycogen/min/mg of protein at 30°C.



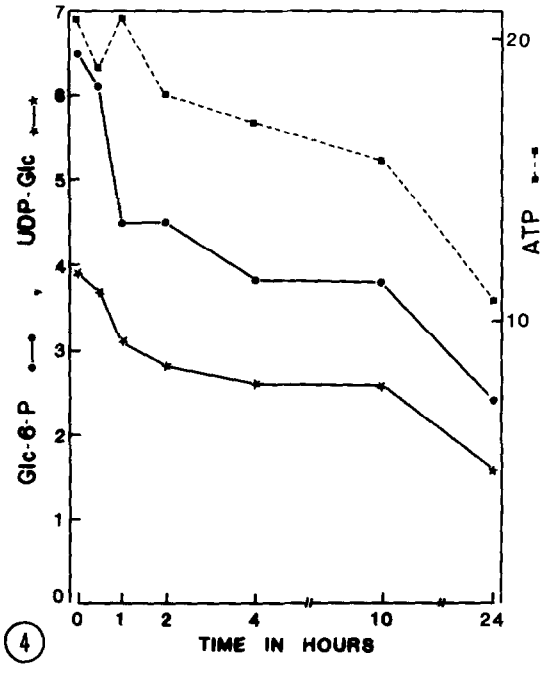


Fig. 3. Effect of glucose starvation on glycogen synthase activity. Experimental conditions were the same as in Fig. 1. Glycogen synthase activity was measured without (□) or with (■) 6 mM glucose-6-P, using the method described by Wang et al. (13). Activities are expressed as nanomoles of UDP-Glucose incorporated into glycogen/min/mg of protein at 30°C.

Fig. 4. Effect of glucose starvation on intracellular concentrations of ATP, glucose-6-P and UDP-Glucose. Experimental conditions were the same as in Fig. 1. Intracellular concentrations of ATP (■), glucose-6-P (●) and UDP-Glucose (★) were determined in deproteinized cell homogenates using enzymatic methods (see materials and methods). Concentrations are expressed in nanomoles per mg of cell protein.

At zero time, phosphorylase a exhibits a low activity, with an a/a+b ratio ranging from 0.07 to 0.13 which indicates that the enzyme is mainly in its inactive dephosphorylated form. Within the minutes following glucose starvation, phosphorylase a increases rapidly and reaches its maximum activity after 1 hour. At this moment of the experiment, about 85 percent of the enzyme is in the active form. Later on, phosphorylase a activity decreases to represent 40 percent of the total activity ; this ratio stays at this value until the end of the experiment.

Effect of glucose starvation on glycogen synthase activity (Fig. 3).

By contrast to glycogen phosphorylase, total (a+b) glycogen synthase activity (assayed in presence of 6 mM glucose-6-P) is unstable, showing an important and regular fall in activity after 4 hours of glucose starvation. At the end of the experiment (24 hours) glycogen synthase a+b activity is found to be only the half of the initial one.

At zero time, before the effect of glucose removal is effective, the glycogen synthase a form activity is found to be low, which is in agreement with the previous results dealing with standard cultures (5).

During the first hour of glucose deprivation, this activity decreases and stays very low, then increases to be maximum after 4 hours (a+/a+b ratio = 0.52) and finally falls down again for longer period of starvation.

Effect of glucose starvation on intracellular concentrations of ATP, glucose-6-P and UDP-glucose (Fig. 4).

ATP content of HT 29 cells is found to remain constant (20 nanomoles/mg of protein) during the first 2 hours of glucose starvation. For longer period of time, the ATP pool undergoes a regular but modest loss, in such a way that at the end of the experiment (24 hours) the cells suffered a 50 percent reduction of its ATP content.

When considering glucose-6-P and UDP-glucose, similar evolution patterns are observed. Starting at 6.8 and 4 nanomoles per mg of protein, respectively, glucose-6-P and UDP-glucose concentrations are reduced by the half after 24 hours of glucose deprivation. However, by contrast to what is found for ATP content, most of the drop in glucose-6-P and UDP-glucose concentrations is observed within the first hour of starvation.

DISCUSSION

When deprived of carbohydrate, the HT 29 cells are able to mobilize their glycogen stores. The glycogenolysis starts within the few minutes following glucose deprivation and is achieved within 4 hours.

The increase in glycogen phosphorylase a activity observed after glucose removal, indicates that this enzyme is largely responsible for glycogen mobilization in these cells. By contrast to what was previously thought (17), the lysosomal hydrolytic pathway for glycogen degradation does not seem to be involved since addition of Chloroquine (a lysosomotropic agent which inhibits the lysosomal function) or Acarbose (a glucosidase inhibitor) do not modify significantly the rate of glycogenolysis (unpublished results). During the first hour of starvation, glycogen phosphorylase activation is concomitant with a decrease in glycogen synthase a, while the total activity remains constant.

After the second hour of glucose starvation, an important decrease in total glycogen synthase activity is observed. As glycogen synthase is tightly associated with the glycogen molecule (18), one can propose that the drop in activity is biophysically related to the decreased number or lower size of glycogen molecules. Such a correlation between total glycogen synthase activity and glycogen content has been already found in the liver of starved/refed rats (19) and in the liver of gsd/gsd rat strain lacking the hepatic phosphorylase kinase (20).

The loss of total glycogen synthase activity coincides with an important increase of the active form of this enzyme ; a similar increase has been observed in liver of rats fasted for more than 12 hours (21). Up to the present time, this is the only experimental situation with glycogen synthase being in its a form, which indicates that glycogen synthase phosphatase is present in HT 29 cells and can dephosphorylate glycogen synthase b under "in vivo" conditions.

It must be pointed out that the activation of glycogen synthase occurs although glycogen phosphorylase is still largely in its active form, which indicates that the glycogen synthase dephosphorylation is not inhibited by phosphorylase a. In this respect, the integrated control system of phosphorylase and synthase is somewhat similar to what has been described in the skeletal muscle and differs from what is proposed for the liver. In HT 29 cells, glycogen itself could be the major factor controlling its own metabolism.

In order to check whether the activation (i.e., dephosphorylation) of glycogen synthase could be due to a dramatic drop of cell ATP content or not, the concentration of this nucleotide as well as of glucose-6-P and UDP-glucose were measured. At zero time, the ATP content in HT 29 cells was similar (20 nanomoles/mg of protein)to what was reported in cultured Chicken fibroblasts (22) Chinese Hamster ovary fibroblasts (23) or Ehrlich ascite cells (24). But, by contrast to what was observed in several tumor cells by Demetrakopoulos et al. (25),HT 29 did not suffer a drastic lowering of ATP content even when starved for 24 hours. We do not have any non speculative explanation for this difference, however it must be noted that these authors used growing cells (monolayers at a density one half to near confluence or cells in suspension) instead of quiescent cells. The possibility that the growing state might explain this difference is under investigation.

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