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Glycogen Metabolism in Human Skin Fibroblasts. Influence of Maltose on the Activity of Acid α-1,4-Glucosidase (38932)

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Acid α-1,4-glucosidase, (EC 3.2.1.2.0), a lysosomal enzyme, exhibits both hydrolase and transferase activities. The enzyme is involved in hydrolytic release of glucose from α-1,4-linkages of maltose and glycogen and in the transfer of glucose derived from maltose to glycogen (1). Deficiency of acid α-1,4-glucosidase in humans results in a pathologic disorder characterized by glycogen accumulation in virtually all tissues, massive cardiomegaly, and cardiorespiratory failure. The syndrome is inherited as an autosomal recessive condition and is commonly known as Cori Type II glycogenosis or Pompe's Disease (2, 3).

The circumstances under which α-1,4-glucosidase functions as a hydrolase or as a transferase have not been well defined. Investigations on the hydrolytic function of α-1,4-glucosidase reported herein are based on observations that the enzyme is active in human skin fibroblasts in culture and that glycogen is stored by these cells when sufficient glucose is available in the medium (4, 5). The primary purpose of this study was to investigate the effects of added maltose to medium on cellular glycogen (while measuring changes in glucose concentration in medium) and the specific activity of α-1,4-glucosidase in human skin fibroblasts in monolayer culture derived from normal subjects and patients with Cori Type II glycogenosis.

Materials and Methods. Subculture and maintenance of fibroblasts. One group of fibroblasts in culture was derived from explants of skin from a normal 15-yr-old female (C. L.) and another from an aborted phenotypically normal human male fetus of approximately 12 wk gestation. Fibroblasts in culture from patients with Cori Type II glycogen storage disease (D. W., a 9-mo-old female, and J. G., a 3-mo-old female) were derived from skin biopsy specimens taken from the intrascapular region.

Eagle's Minimum Essential Medium (6) with either Hanks' salts (HMEM) (7) or Earle's salts (EMEM) (8) in the form of dry powder (Schwarz Bioresearch, Orangeburg, New York 10952) was reconstituted, and then filtered in 10 liter quantities through 0.22 μm Millipore filters (Millipore Corp., Bedford, Massachusetts 01730). The medium was supplemented just prior to use with 10% (v/v) fetal calf serum, 50 U/ml each of penicillin and streptomycin (Microbiological Associates, Bethesda, Maryland, 20014) and 2.5 μg/ml amphotericin B (*Fungizone*, E. R. Squibb and Sons, Inc., New York, NY 10001) unless otherwise specified.

Confluent monolayers were subcultured by treating the cells with 0.25% trypsin in bicarbonate-buffered Earle's balanced salt solution (final pH 7.6) and triturated with HMEM. The cells were allowed to reattach to the glass surface of a 120 cm^2 flask in HMEM for 24–48 hr. The medium was then changed to EMEM, and the cells were maintained in this medium with changes every 4 days except as otherwise noted in particular experiments (see Figs. 1 and 2).

Enzyme assays. The cell surfaces were washed twice with 10 ml cold phosphate buffered saline (PBS) as described by Dulbecco and Vogt (9). The fibroblasts were scraped off the glass surface with a rubber policeman into 10 ml cold PBS and centrifuged at 500*g* for 2–3 min. Cells were then resuspended in 0.25 *M* sodium acetate buffer,

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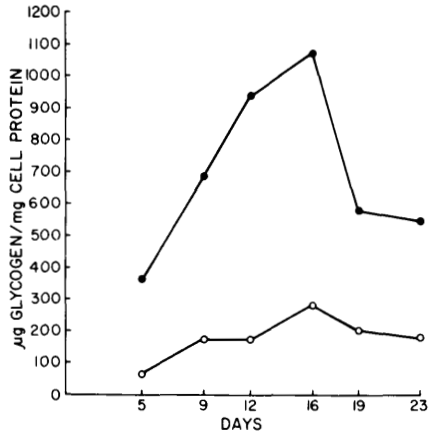

FIG. 1. Glycogen accumulation in normal skin fibroblasts (○——○) and in Cori Type II glycogen storage disease skin fibroblasts (●——●) incubated with EMEM medium supplemented with 100 mg/100 ml of maltose.

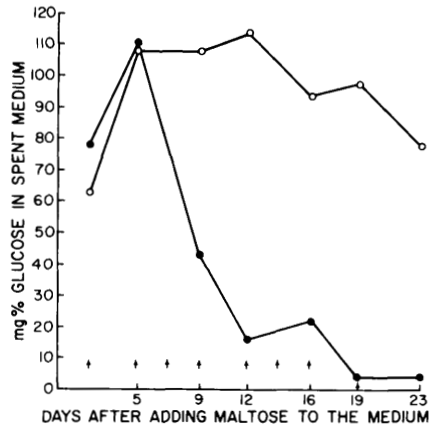

FIG. 2. Glucose concentration in medium supplemented initially with 100 mg/100 ml of maltose after incubation with normal skin fibroblasts (○——○) and with Cori Type II Glycogen Storage Disease skin fibroblasts (●——●). Arrows indicate time of medium change with EMEM supplemented with 100 mg/100 ml of maltose.

pH 4.0, and disrupted by freeze-thawing six times in an acetone–dry ice bath. The suspension was agitated for 5 sec on a Vortex mixer (Scientific Industries, Inc., New York 10001) between freezings, centrifuged at 2000*g* for 10 min, and assayed immediately for α-1,4-glucosidase activity. Protein content was determined in all experiments by the method of Lowry and coworkers (10). Acid α-1,4-glucosidase was assayed by a modification of the procedure of Leathwood and Ryman (11), and aryl sulfatase A activity was determined by the method of Kaback and Howell (12). In assaying acid α-1,4-glucosidase activity, a 50 μl aliquot of supernatant solution containing from 10 to 50 μg of protein was added to 0.2 ml of 0.25 *M* sodium acetate buffer, pH 4.0, containing 11.6 μ*M* of maltose. After incubating the mixture at 37° for 60 min, the reaction was stopped by heating for 2 min in a boiling water bath. The mixture was then cooled, and 0.15 ml 0.05 *M* $Ba(OH)_2$, 0.15 ml 0.05 $ZnSO_4$, and 0.45 ml saturated benzoic acid were added. The glucose released by hydrolysis from maltose by endogenous acid α-1,4-glucosidase was measured using an automated fluorometric procedure for glucose (13). The activity of α-1,4-glucosidase in the supernatant derived from the cells was expressed as micrograms of glucose hydrolyzed/hour at 37°/mg protein.

Glycogen determination. Fibroblasts in culture were washed and cells were scraped into cold PBS as described in the previous section. After centrifugation at 500*g* for 2–3 min, the supernatant solution was decanted. Cells were then disrupted in cold deionized water with a model G S-110 ultrasonic oscillator (T. M. Branson Instruments, Inc., Melville, NY 11746) at the maximum setting for 20 sec. The sonicate was heated in a boiling water bath for 2 min, cooled in an ice bath, and assayed immediately for glycogen and protein, or the sonicate was stored at −20° until the assay could be performed.

Glycogen content of the cell sonicate was determined by a modification of the procedure of Huijing (14). An aliquot of 0.2 ml of the sonicate (containing from 8 μg to 80 μg protein) was mixed with 0.2 ml 0.2 *M* sodium acetate buffer, pH 6.0, containing 2.5 U/ml α-1,4-glucosidase (Boehringer-Mannheim Corp., Freehold, NJ 07728) and incubated in a water bath at 37° for 90 min. After incubation, 0.6 ml saturated benzoic acid was added to each incubation mixture, and glucose was then determined. Solutions of glycogen at various concentrations served as standards, and all tests were done in duplicate. Glycogen content was expressed as micrograms of glycogen/mg protein.

Results. Human skin fibroblasts from a

normal subject (C. L.) in passage 10 and skin fibroblasts from a patient with Cori Type II glycogen storage disease (D. W.) in passage 8 were harvested and compared for glycogen content. Fibroblasts at confluency (day 5) from the normal subject contained 79 μg glycogen/mg cell protein, whereas fibroblasts at confluency (day 5) from the patient with Cori Type II glycogenosis contained 409 μg glycogen/mg protein. Studies were also conducted on fibroblasts derived from a normal human male fetus of approximately 12 wk gestation and from a 3-mo-old female (J. G.) with Cori Type II glycogenosis, and the results were qualitatively and quantitatively similar to those obtained on the cells from the normal subject (C. L.) and from the patient (D. W.).

To test the influence of maltose on glycogen deposition in fibroblasts in culture, we incubated the cells in EMEM containing 100 mg/100 ml maltose, 5% (v/v) fetal calf serum, 50 U/ml each of penicillin and streptomycin, and 2.5 μg/ml amphotericin B. Medium was changed every other day, and the amount of glycogen in the cells was determined on days 5, 9, 12, 16, 19, and 23 after addition of maltose. Results are shown in Fig. 1. Normal skin fibroblasts reached confluency by the fifth day after subculture and were observed to have an increasingly distended appearance from day 5 to day 16, during which time there was an increase in glycogen concentration from 79 μg/mg cell protein to 300 μg/mg cell protein (20 μg glycogen/mg protein/day). Skin fibroblasts derived from patients with Cori Type II glycogenosis, however, appeared to grow at a somewhat slower rate than the normal fibroblasts and were quite distended when the cells reached confluency at days 5 or 6. Glycogen content in these cells increased from day 5 to day 16 from 409 μg/mg cell protein to 1100 μg/mg cell protein (63 μg glycogen/mg protein/day). By day 19, the concentration of glycogen had decreased to 71% (212 μg/mg cell protein) of the level at day 16 in fibroblasts derived from skin of normal subjects and to 46% (506 μg/mg cell protein) of the level at day 16 in fibroblasts from Cori Type II glycogen storage disease as shown in Fig. 1. It is interesting to note that in spite of the higher glycogen levels in the cells derived from the patients with Cori Type II glycogenosis, glucose levels in the medium decreased markedly, suggesting that a marked uptake of glucose occurred which was persistent over the period of time that maltose was added repeatedly at the designated times in a concentration of 100 mg% in the medium (see Figs. 1 and 2).

In other experiments, it was shown that maltose added to the cultures had a pronounced effect on the specific activity of α-1,4-glucosidase. In these studies medium (EMEM) containing 300 mg/100 ml maltose was incubated with fibroblasts from normal subjects and from patients with Cori Type II glycogenosis. Cells were subcultured at confluency, and enzyme activity of the cells was measured over a period of 46 days. The relative specific activity of acid α-1,4-glucosidase of the normal cells (C. L.) grown in the presence of maltose increased nine- to tenfold over the control cells cultured in medium without added maltose. There was no comparable increase in acid α-1,4-glucosidase activity detected in fibroblasts derived from the skin of the patients with Cori Type II glycogen storage disease. Activity of aryl sulfatase A, another lysosomal enzyme (used as an additional check on lysosomal integrity under the experimental conditions imposed), was shown to be independent of cell population density. Also, activity of aryl sulfatase A observed under experimental conditions shown in Fig. 3 was comparable in normal skin fibroblasts and in fibroblasts derived from patients with Cori Type II glycogenosis.

Discussion. The initial response to an increase of maltose in medium in normal fibroblasts is an increase in intracellular glycogen content. When α-1,4-glucosidase is activated in the first 1–3 days following subculture, the increase in activity is accompanied by hydrolysis of maltose in the medium to glucose. As a result, the level of free glucose in medium of normal fibroblasts is maintained while glycogen accumulates within the cell as shown in Figs. 1 and 2. When α-1,4-glucosidase activity decreases from day 16 to day 23, it would appear that less maltose is converted to glucose, and the concentration of free glucose in medium

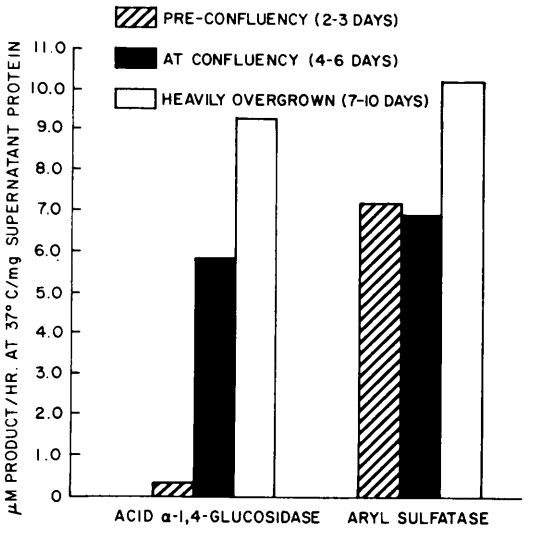

FIG. 3. Specific activity of acid α-1,4-glucosidase and aryl sulfatase A in normal human skin fibroblasts following subculture during the rapid growth phase (2–3 days), at confluency (4–6 days), and in a compact, overgrown state (7–10 days). Enzyme activities were measured in the absence of added maltose.

decreases. There is no detectible acid α-1,4-glucosidase activity in Cori Type II glycogenosis cells, and, as expected, little or no conversion of maltose to glucose occurs. Therefore, a decrease in concentration of glucose in medium in Cori Type II glycogenosis cells probably reflects actual glucose utilization by the fibroblasts.

In our experiments glucose concentration of spent medium continues to decrease in normal fibroblasts despite frequent medium changes. DiMauro and Mellman have shown that repeated additions of medium supplemented with 10% fetal calf serum results in accumulation of glycogen in cells (4, 5). This is most likely mediated through an effect of insulin and/or possibly other factors in serum on the rate of glycogen storage in fibroblasts in culture, especially in those depleted of glycogen by glucose starvation. Insulin probably enhances glycogen deposition in cultured fibroblasts by facilitation of glucose entry into cells, but, in addition to stimulating transport in human diploid fibroblasts, insulin also enhances glucose utilization and lactate production via glycolysis.

Fibroblasts derived from normal subjects persist in uptake of glucose from the fifth to the twelfth day after addition of maltose to the medium, but at day 16 (when normal cells show a marked decrease in glycogen concentration/mg protein) there is an increase in the amount of glucose in the medium. Beginning at day 16 the rapid decrease in glycogen concentration in Cori Type II glycogen storage fibroblasts is not due to activation of acid α-1,4-glucosidase since these cells show no demonstrable activity of this enzyme at any time. Studies by DiMauro and Mellman show an almost identical pattern of decrease in glycogen concentration in Cori Type II fibroblasts to that which we observed except for a slight variation in the time course (5). Our preliminary findings by cell fractionation techniques and electron microscopy show that glycogen did not accumulate in lysosomes after day 16. The question arises whether there is extralysosomal breakdown of the accumulated glycogen in fibroblasts derived from patients with Cori Type II glycogen storage disease which could be explained by activation of some other hydrolase or glycogenolytic enzyme in the cytoplasm.

The cell population density of fibroblasts derived from normal human skin apparently influences the specific activity of acid α-1,4-glucosidase rather markedly but not that of aryl sulfatase A, another lysosomal enzyme. When fibroblasts reach confluency at 4–6 days there is a 20-fold relative increase in specific activity of α-1,4-glucosidase over the preconfluency period (2–3 days) while the specific activity of aryl sulfatase A remains constant as seen in Fig. 3. Over 7–10 days following subculture the specific activities of both α-1,4-glucosidase and aryl sulfatase A increase about 1.5-fold.

Summary. Maltose added to medium in a concentration of 100 mg/100 ml enhances the hydrolytic activity of acid α-1,4-glucosidase in skin fibroblasts in culture derived from normal human subjects, but under the same conditions maltose has no demonstrable effect on the enzyme in fibroblasts derived from skin of patients with Cori Type II glycogenosis. Upon addition of maltose to Cori Type II fibroblasts there is a marked decrease of glucose in medium overlying the cells with a concomitant marked increase in glycogen content of the

same cells. Glycogen decreases in a similar manner in both normal and mutant cell types after 16 days in culture. This apparent utilization or degradation of glycogen in Cori Type II fibroblasts in culture is difficult to explain unless only a proportion of the polysaccharide accumulates within lysosomes. We propose, therefore, that extralysosomal glycogen which accumulates in fibroblasts from Cori Type II glycogenosis may be more easily degraded by glycogenolytic cytoplasmic enzymes, particularly in cells grown for as long as 2 wk in glucose-starved media.

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