# LYSOSOMAL GLYCOSIDASE ACTIVITY IN HUMAN FIBROBLASTS IN CULTURE

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UDC 612.79.015.16:577.
152.11/-085.23-088.1

KEY WORDS: fibroblast culture; lysosomal glycosidases.

Cutaneous fibroblasts in culture are used for the diagnosis of many hereditary diseases and, in particular, glycosidoses which develop as a result of disturbances of enzymic breakdown of conjugated saccharides [1]. It will be clear that the more successful biochemical diagnosis of these diseases requires precise data on glycosidase activity in fibroblasts in culture. Meanwhile the obtaining of such data is beset by technical difficulties, and in particular, by the fact that cells in transplantable cultures exist in two interchanging physiological states: intensive multiplication in the logarithmic phase of growth and proliferative rest in the confluent monolayer [2].

In the investigation described below changes in specific activity of three lysosomal hydrolases — β-D-galactosidase (β-Gal, E.C. 3.2.1.23), α-L-fucosidase (α-Fuc, E.C. 3.2.1.51), and N-acetyl-β-D-hexosaminidase (β-Hex, E.C. 3.2.1.52) — were studied in relation to the phase of growth of the monolayer and times of passage of embryonic and postnatal fibroblasts, and the importance of some methods of obtaining enzyme preparations also was determined. Data on the effect of the first group of factors are contradictory [3-6], and the role of other factors is not mentioned in the known literature. Choice of these enzymes for investigation was determined by the fact that their hereditary deficiency leads to the development of corresponding enzymopathies [1].

## EXPERIMENTAL METHOD

Cutaneous embryonic fibroblasts of strain 814 were obtained from the cell bank of the Institute of Medical Genetics, Academy of Medical Sciences of the USSR, and postnatal fibroblasts of strain 101 were obtained from the cell bank of the Institute of Rheumatism, Academy of Medical Sciences of the USSR. The fibroblasts were grown in 50-ml flasks on a mixture of medium 199 and Eagle's medium (1:1) with the addition of glutamine, 20% bovine serum, and antibiotics. The seeding density of strain 814 was $1.2 \cdot 10^4$ cells, and of strain 101, $2 \cdot 10^4$ cells/cm$^2$. Before removal the cells were washed with a mixture of physiological saline and phosphate-citrate buffer, pH 4.5 (9:1) and were removed from the substrate with 0.25% trypsin solution (Spofa, Czechoslovakia), and in some experiments by mechanical scraping. The number of cells in each vessel was counted in a Goryaev's chamber, after which the cells were sedimented by centrifugation at 3500g for 30 min. The cell residue was frozen once in 100-200 µl of 0.05 M phosphate-citrate buffer. After thawing, the cell preparations were homogenized in the cold in a Teflon-glass homogenizer for 20 sec and used for determination of specific activity of the above-mentioned glycosidases, using the corresponding fluorogenic 4-methylumbelliferyl glycosidases: β-D-galactopyranoside, α-L-fucopyranoside, and N-acetyl-β-D-glucopyranoside. Enzyme activity was determined as the quantity of free 4-methylumbelliferyl (4-MUF) formed during hydrolysis of the glycosides. The incubation sample (final volume 250 µl) contained 100 µl of the corresponding substrate (final concentration 0.5 mM, but 0.26 mM for β-Hex), made up in 0.15 M phosphate-citrate buffer (pH 4.5). The sample was made up to the final volume with the same buffer. The reaction was stopped by adding 250 µl of 0.4 M glycine-NaOH buffer, pH 10.5, to the incubation sample. The quantity of free 4-MUF was judged from the change in fluorescence, on a Soviet fluorometer of the 3F-3MA type ($\lambda_{exc}$ = 365 nm, $\lambda_{fl}$ = 450 nm). The unit of glycosidase activity was taken to be the quantity of each enzyme which hydrolyzes 1

Laboratory of the Biochemical Basis of Hereditary Diseases, Institute of Biological and Medical Chemistry, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR V. N. Orekhovich.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 98, No. 11, pp. 550-553, November, 1984. Original article submitted January 24, 1984.

0007-4888/84/9811-1501$08.50  

nanomole of substrate in 1 h under standard conditions. Specific activity was characterized as the number of units of each enzyme, expressed per milligram protein or per $10^6$ cells when a near-saturation concentration of the corresponding substrates was created in the samples. In the case of β-Hex, determination of which was limited by the insufficient quantity of substrate, its saturating concentration was created only in random tests, and a corresponding calculation was done for the rest. Protein concentration was determined by Lowry's method [9].

## EXPERIMENTAL RESULTS

With an increase in the time after subculture at the confluent monolayer stage glycosidase activity increased in both types of cells (Table 1). However, the intensity of these changes depended on the type of fibroblasts (embryonic or postnatal), the time after subculture, and the method used to calculate enzyme activity per protein or per cell. From the second until the 11th days of growth of postnatal fibroblasts there was a roughly equal increase in all glycosidase acitvities: The increase was most marked when activity was expressed per cell (60-70%) and it was much lower when calculated relative to protein (30-40%). This disparity, when enzyme activity was expressed differently, can be explained by an increase in protein content in the cell at the monolayer stage (from 462 ± 70 to 544 ± 46 μg/$10^6$ cells). For embryonic fibroblasts the change in enzyme activity was less homogeneous: By the 6th day of growth an increase in α-Fuc and β-Gal activity was observed by 80 and 90%, respectively, when calculated per cell, and by 37 and 72% when calculated relative to protein. With an increase in the time after subculture to 7 days, changes in glycosidase activities were more clearly defined. Under these circumstances the maximal increase (by 177 and 278%, respectively) was observed for β-Gal and α-Fuc, whereas the increase for β-Hex was 70% (expressed per cell).

The content of cellular protein was increased (from 571 ± 56 to 637 ± 28 μg/$10^6$ cells) and this led to lower values of specific activity of the glycosidases when calculated relative to protein.

Data on enzyme activity in the two types of fibroblasts during a series of passages are evidence (Table 2) that these parameters change only a little between several successive passages. Meanwhile, in late passages of embryonic cells, an increase of 200% was observed in β-Gal and α-Fuc activity calculated per cell (or 100% if calculated per protein) compared with the early passages. The protein content in the cell also increased from 392 ± 39 to 571 ± 56 μg/$10^6$ cells. Values of specific glycosidase activity may be distorted not only by an objective factor such as the changing physiological state of the cells in culture, but also by certain technical measures used to obtain the enzyme preparations. The writers showed that mechanical scraping of a thinly packed monolayer leads to a decrease in total α-Fuc and β-Gal activity in the cell preparation by 80-82%. The distribution of protein between cell residue (37%) and supernatant (63%) in this case does not correspond to the distribution of total enzyme activities, and this leads to distortion of the specific glycosidase activities determined with calculation per protein. When cells of a confluent monolayer are scraped, total activity of these enzymes and protein are distributed about equally between cell residue and supernatant. Brief treatment of the cells with trypsin causes virtually no loss of glycosidase activities.

TABLE 1. Specific Activity of Glycosidases of Human Cutaneous Fibroblasts at Different Stages of Monolayer Development (M ± m; n = 3-4)

| Fibroblasts | No. of passage | Time after subculture | β-Hex | | β-Gal | | α-Fuc | |
|---|---|---|---|---|---|---|---|---|
| | | | A | B | A | B | A | B |
| Strain 814 | 36 | 2 | 1569±50 | 781±62 | 123±11 | 61±3 | 7,9±0,6 | 3,9±0,6 |
| | | 7 | 2694±140 | 1411±185 | 322±12 | 169±16 | 29,9±0,6 | 15,7±2,0 |
| | 39 | 2 | 2267±171 | 1307±160 | 191±5 | 110±7 | 9,9±0,4 | 5,7±1,5 |
| | | 6 | 2414±78 | 1536±58 | 330±9 | 210±18 | 13,6±1 | 8,6±0,1 |
| Strain 101 | 9 | 2 | 4152±306 | 1955±290 | 342±26 | 155±10 | 66±2 | 31±3 |
| | | 11 | 5768±113 | 3144±244 | 491±30 | 264±21 | 88±6 | 48±7 |

Legend. Here and in Tables 2 and 3: A) nmoles/h/mg protein, B) nmoles/h/$10^6$ cells. Incubation time 10 min for β-Hex, 1 h for β-Gal, and 4 h for α-Fuc.

TABLE 2. Specific Activity of Glycosidases of Human Cutaneous Fibroblasts at Different Passages (M ± m, n = 3-4)

| Fibroblasts | No. of passage | β-Hex | | β-Gal | | α-Fuc | |
|---|---|---|---|---|---|---|---|
| | | A | B | A | B | A | B |
| Strain 814 | 27 | — | — | 180±10 | 66±6 | 13,3±0,9 | 4,8±0,5 |
| | 29 | — | — | 123±12 | 42±7 | 14,2±3 | 4,8±0,4 |
| | 30 | — | — | 165±18 | 72±6 | 9,9±1,4 | 4,7±0,3 |
| | 37 | 2349±173 | 1185±176 | 305±33 | 151±19 | 15,6±2,8 | 8,0±0,6 |
| | 39 | 2414±78 | 1536±58 | 330±9 | 210±18 | 13,6±1 | 8,6±0,1 |
| | 40 | 2590±154 | 1459±81 | 360±21 | 191±23 | 9,5±1,6 | 5,3±1 |
| Strain 101 | 8 | 6794±548 | 3005±320 | 645±33 | 285±13 | 102±5 | 44±5 |
| | 9 | 5768±113 | 3144±244 | 491±30 | 264±21 | 87±6 | 48±7 |
| | 10 | 6162±219 | 3642±220 | 452±38 | 287±32 | 109±8 | 65±6 |

TABLE 3. Specific Glycosidase Activity Depending on Density of Monolayers (M ± m, n = 3)

| Number of cells in sample $\times 10^6$ (volume of sample 200 µl) | Protein in sample, µg (volume of sample 20 µl) | β-Gal | | α-Fuc | |
|---|---|---|---|---|---|
| | | A | B | A | B |
| 2,44 | 83±1 | 169±5 | 58±2 | 15,0±0,1 | 5,1±1,0 |
| 1,37 | 57±2 | 143±4 | 59±1 | 11,3±0,7 | 4,7±0,5 |
| 0,61 | 20±1 | 59±7 | 19±2 | 3,5±0,7 | 1,2±0,02 |

Homogenization (after thawing) of small numbers of cells in a standard volume of buffer may also give considerably underestimated values for specific activity of lysosomal glycosidases. Table 3 gives data on α-Fuc and β-Gal activity in cells removed from one culture vessel, but differing by two and four times in the quantity in 200 µl of buffer. On homogenization of $0.61 \cdot 10^6$ cells (corresponding to a protein concentration of 1 µg/µl) the value of specific activity determined was 25-30% of values obtained in samples of the same volume but with a larger (2-4 times) number of cells.

The results agree with data in the literature on changes in activity of several lysosomal glycosidases of fibroblasts depending on time after subculture and duration of passage of the cells [3, 4, 6-8, 10, 11]. Activity of the enzymes studied in cutaneous embryonic fibroblasts changed by a greater degree than in postnatal fibroblasts. A change in the physiological state of the cells led to a much greater increase in β-Gal and α-Fuc activity compared with the change in β-Hex activity.

These results are evidence that changes in specific activity of lysosomal glycosidases of cells in different states must be estimated by calculation per number of cells and not per protein, the concentration of which may vary.

When activity of lysosomal glycosidases is determined, in order to discover deviations from normal values it is important to observe a number of conditions when obtaining and processing the cell material. Glycosidase activity must be determined at the same stages of development of the monolayer (for preference in a confluent monolayer, in which it is maximal) and at about the same stages of passage of the cell strains to be compared. It is evidently preferable to remove cells from the substrate by means of trypsin solution, for mechanical scraping may lead to distortion of specific activities. For the same reason, the optimal number of cells must be created in samples for homogenization.

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# EFFECT OF BILE ON AMYLOLYTIC ACTIVITY OF SOME BIOLOGICAL MEDIA

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UDC 612.332.015.1:577.152.321]-06:612.357.2

KEY WORDS: amylase; bile; changes in enzyme activity.

Changes in enzyme activity are among the principal mechanisms of metabolic adaptation *in vivo*. Many of the factors determining the level of catalytic enzyme activity have been elucidated [3]. As regards amylase, which takes part in intestinal digestion, there is evidence that it is affected by activity of bile acids [2] or of whole bile [4], present in the intestine. Components of bile are contained in the internal medium and tissues of the body [1], where their concentration may rise in biliary stasis.

The aim of the present investigation was to discover whether an increase in concentration of components of bile is reflected in amylase activity in these media.

## EXPERIMENTAL METHOD

The effect of bile on the amylolytic activity of human saliva, blood plasma and homogenates of the pancreas and mucosa of different parts of the small intestine of rats was investigated *in vitro*.

Blood and tissues of these organs were obtained from the rats after decapitation. The pancrease and the intestinal mucosa, washed to remove chyme, were homogenized with a Teflon pestle in Ringer's solution, pH 7.0, at the rate of 1 ml of liquid to 100 mg tissue. Plasma was obtained by centrifugation of blood mixed with 5% NaCl solution in the ratio of 1:50; saliva was diluted before analysis with Ringer's solution in the ratio of 1:20,000. All media for study were prepared at 0-4°C.

Amylase activity was determined by Ugolev's method [5] and expressed in milligrams starch hydrolyzed per minute per milliliter (of saliva or blood plasma) or per gram of tissue (pancreas and intestinal mucosa). Bile was added to the test media in doses of 0.1, 0.2, 0.5, and 1 ml. An increase in the concentration of bile acids by 2.5, 5, 12, 5, and 25 mg%, respectively, was created in this way in the medium.

The numerical data were subjected to analysis by the Student–Fisher method.

## EXPERIMENTAL RESULTS

Addition of bile to a medium in which enzymic hydrolysis of starch takes place was shown to accelerate the course of the reaction. Activation was not associated with the presence of amylase in the bile itself (its activity was accounted for in the calculations).

The presence of bile had a stimulating action on amylase activity, which differed in its origin. An increase in activity of amylase synthesized by cells of the salivary glands, pancreas, and intestinal mucosa was observed. Meanwhile, with the same quantity of bile, the increase in amylase activity differed in different media. On addition of 0.2 ml of bile, amylase activity in the blood plasma increased by 280%, in saliva by 146%, in homogenate of the pancreas by 152%, in the jejunal mucosa by 114%, and in the ileal mucosa by 150%.

The degree of increase in amylase activity depended on the quantity of bile added to the medium. With a gradual increase in the quantity added, the maximum of the effect was observed

Department of Normal Physiology, Tashkent Medical Institute. (Presented by Academician of the Academy of Medical Sciences of the USSR A. D. Ado.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 98, No. 11, pp. 553-555, November, 1984. Original article submitted April 3, 1984.

 0007-4888/84/9811-1504$08.50 