Fabry's Disease: Differentiation between Two Forms of α-Galactosidase by Myoinositol

Abstract. *Myoinositol appears to be a competitive inhibitor of α-galactosidase activity in fibroblasts of normal human skin but not of the residual α-galactosidase activity of fibroblasts obtained from patients with Fabry's disease. It is suggested that normal fibroblasts contain two α-galactosidases, only one of which is present in cells from patients with Fabry's disease, and that these enzymes can be distinguished by their different Michaelis constants, rates of heat inactivation, and responses to the inhibitor myoinositol.*

Fabry's disease is an X-linked recessively inherited disorder, characterized by deposition of the glycolipid ceramide trihexoside in various organs of the body; depositions are frequently associated with blood vessels. It can give rise to a characteristic skin lesion, angiokeratoma, and the patients may die in middle age of a cerebral artery hemorrhage or renal failure (*1*). There is a specific defect of ceramide trihexosidase in the gut mucosa of these patients (*2*), and a defect of α-galactosidase has been detected in circulating leukocytes and cultured skin fibroblasts (*3*). In contrast to the findings in the latter report, we find that the deficiency of this enzyme is not complete but that residual enzyme activity is between 10 and 20 percent of that found in normal cells. There are three possible explanations for this. (i) Fabry's disease is associated with a regulatory gene defect that reduces the quantity of the normal enzyme being formed. (ii) The residual enzyme activity arises from a structural gene mutation modifying the structure of the normal enzyme. (iii) Two or more isoenzymes for α-galactosidase exist, the principal one of which is missing in Fabry's disease. In that case the missing enzyme might represent the true ceramide trihexosidase.

We have investigated these three possibilities by studying the reaction of the enzyme with the artificial substrate 4-methylumbelliferyl-α-galactoside and have used the techniques of determination of Michaelis constant (K_m), heat inactivation, and specific inhibition of the enzyme. Three patients with Fabry's disease were studied. One patient had typical angiokeratoma, and one had no angiokeratoma but a significant family history of Fabry's disease. This patient developed renal failure with the renal and urinary glycolipid findings characteristic of Fabry's disease. The third patient had no angiokeratoma or renal failure but did have proteinuria and the renal histology and urinary glycolipids characteristic of Fabry's disease. Details of these last two patients have been reported (*4*). Since myoinositol was a selective inhibitor of various plant α-galactosidases when *p*-nitrophenyl-α-galactoside was used as a substrate (*5*), we studied the effect of this inhibitor on the α-galactosidase activity of cultured skin fibroblasts from the patients with Fabry's disease and from normal control cells. The total enzyme activity of these cell strains and their corresponding K_m values are shown in Table 1.

The rate of heat inactivation of α-galactosidase in these cell strains was measured. The enzyme from the normal cell lines was rapidly inactivated at 51°C over a period of 60 minutes. After longer periods of heating, the rate of heat inactivation was much slower and closely paralleled that found for the residual enzyme activity in the cell strains from patients with Fabry's disease. The K_m values were again determined on the residual α-galactosidase activity present in a normal cell strain after heat inactivation, and the K_m of this residual enzyme was 19.5 m*M*, as compared to 4.0 m*M* for enzyme activity before heat treatment.

The inhibition of α-galactosidase activity in the presence of myoinositol was then studied. Myoinositol appeared to be a competitive inhibitor of the normal enzyme (Fig. 1) and was most effective at 500 m*M*, the highest concentration of substrate compatible with keeping the substrate in solution. In contrast, the enzyme activity present in cell extracts from patients with Fabry's disease was not inhibited by myoinositol at any inhibitor concentration; in fact it seemed to have a mild stimulatory effect on some cell strains. Experiments combining heat inactivation and myoinositol inhibition were then carried out; an example is shown in Fig. 2. Myoinositol is an inhibitor of α-galactosidase obtained from a normal cell strain, but during the period of heat inactivation, when the α-galactosidase activity is decreased, the effect of myoinositol as an inhibitor also decreases; after 60 minutes of heat inactivation no inhibition of the enzyme is observed. This could be explained by the hypothesis that all the heat-labile enzyme is destroyed, leaving the heat-stable enzyme characteristic of Fabry's disease, which is not inhibited by myoinositol. The residual enzyme activity of the normal cell extract is quantitatively similar

Table 1. α-Galactosidase activity and corresponding K_m values of cultured fibroblasts. Cultured cells were sonicated (Branson Sonifier), and the substrate, 4-methylumbelliferyl-α-galactoside, was dissolved in 0.1*M* acetate buffer (*p*H 5.0). The reaction mixture, consisting of 20 μl of sonicated material and 180 μl of substrate (1 μmole, 5 m*M* final concentration) was incubated at 37°C for 2 hours. The reaction was stopped by addition of 2.8 ml of glycine buffer (*p*H 10.5), and the fluorescence was measured in an Aminco Bowman spectrophotofluorimeter (exciting wavelength, 360 nm; fluorescence, 450 nm) and compared with 4-methylumbelliferone as standard; K_m values were determined for extracts of normal cells (0.5 to 5 m*M* substrate) and for cell extracts of patients with Fabry's disease (5 to 25 m*M* substrate).

Enzyme activity (nanomoles of substrate hydrolyzed per milligram of protein per hour)		K_m (m*M*)	
Normal	Fabry's disease	Normal	Fabry's disease
	7.4	3.34	22.2
51.2 ± 18.1*	5.9	2.89	28.6
	7.7	3.95	14.3

* Mean and standard deviation from 14 determinations carried out on eight different strains of normal cells.

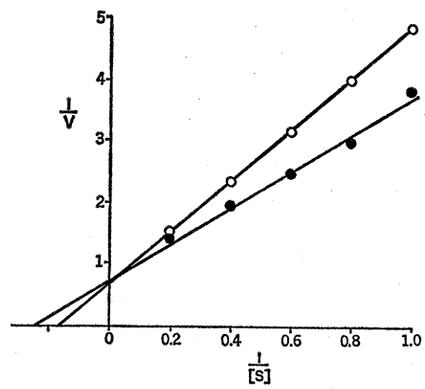

Fig. 1. Lineweaver-Burke plot showing myoinositol as a competitive inhibitor of α-galactosidase in sonicated normal skin fibroblasts. The enzyme was assayed as described in the legend to Table 1. The reaction velocity, *V*, is expressed as nanomoles per milligram of protein per hour. The reciprocal of this velocity has been multiplied by 10^2 to obtain the values on the vertical axis. The substrate concentration, [*S*], is millimolar. The concentration of the inhibitor, myoinositol, was 100 m*M*. The K_m calculated for the normal enzyme was 3.95 m*M* and that for the inhibited enzyme was 6.0 m*M* (●—●, α-galactosidase activity without myoinositol; ○—○, α-galactosidase activity with myoinositol).

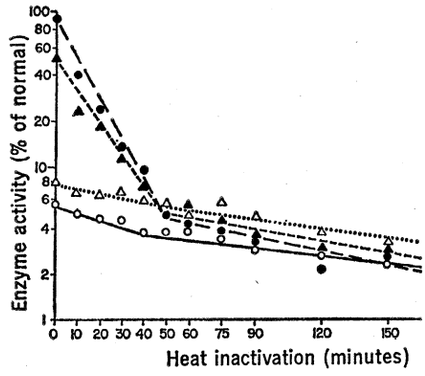

Fig. 2. Effect of heat inactivation and myoinositol on α-galactosidase activity of sonicated preparations of cultured fibroblasts from a normal subject and those from a patient with Fabry's disease. Heat inactivation of the enzyme in the cell preparation was carried out at 51°C for various periods of time. The heat-inactivated enzymes were then mixed with the substrate in 0.1*M* acetate buffer (*p*H 5.0), incubated as described in Table 1, with 5 m*M* substrate and 500 m*M* inhibitor (●—●, α-galactosidase activity of normal cells; ▲—▲, of normal cells, myoinositol added; ○—○, of cells from patient with Fabry's disease; and △—△, of cells from patient, myoinositol added).

to that found in the cell extracts from patients with Fabry's disease. Myoinositol had a mild stimulatory effect on patients' cell extracts as compared with the inhibitory effect on the normal cell extract (Fig. 2). Myoinositol had no inhibitory effect on the β-galactosidase activity in fibroblasts.

These observations are not compatible with the hypothesis of a regulator gene defect nor of a structural gene variation giving rise to an atypical enzyme. They do support the hypothesis, proposed on the basis of K_m data and electrophoretic separation of two different enzymes from leukocytes (*6*), that there is a separate heat-labile enzyme that is absent in the X-linked condition Fabry's disease. The present experiments demonstrate that the residual α-galactosidase enzyme found in fibroblast cells of patients with Fabry's disease is characterized by its higher K_m, its resistance to heat inactivation, and the lack of inhibition by myoinositol.

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References and Notes

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Flavonols: Pigments Responsible for Ultraviolet Absorption in Nectar Guide of Flower

Abstract. *The petals of the black-eyed susan (Compositae:* Rudbeckia hirta*) contain three flavonol glucosides (6,7-dimethoxy-3',4',5-trihydroxyflavone-3-O-glucoside, patulitrin, and quercetagetin). These compounds, which show intense spectral absorption at 340 to 380 nanometers, are restricted in distribution to the petal bases, which are ultraviolet absorbing as a result. Such ultraviolet-absorbing petal zones, known as "nectar guides," are invisible to us, but are visible and of orientation value to the pollinating insect that lands on the flower in search for food. This is the first time that ultraviolet absorption in a nectar guide has been interpreted in chemical terms. In view of the widespread occurrence of flavonols in flowers, it is suggested that these pigments serve specifically for demarcation of ultraviolet petal patterns visible and relevant to insects.*

The petals of flowers commonly show differential color markings at their base. Such markings have long been known as nectar guides (*1*). Located centrally on the flower, near the nectaries, the guides cue pollinating insects to the presence of adjacent food (*2, 3*). Nectar guides are frequently invisible to man. Consisting of ultraviolet-absorbing patches, they are discernible only to insects such as honey bees, whose visual sensitivity extends into the near-ultraviolet region of the solar spectrum (*3–5*). Although much is known about the floral pigments responsible for the visible colors of flowers, the chemical basis of ultraviolet absorption in nectar guides has remained unexplained. Evidence suggested that phenolic pigments were involved (*6*), but the pigments were not characterized. We have now demonstrated that ultraviolet absorption in the nectar guide of a composite flower, the familiar black-eyed susan (*Rudbeckia hirta*), is attributable to a mixture of flavonol glucosides.

To the human eye, the petals of *Rudbeckia* appear evenly yellow (Fig. 1A). Ultraviolet photography reveals the darkly absorbing nectar guides, which encompass the entire basal halves of the petals (see Fig. 1B). Reflection spectra of the base and apex of a petal (see Fig. 2A) show the two regions to be closely similar in the visible portion of the spectrum, but to differ sharply in the near ultraviolet (*7*).

Separate extraction, by prolonged soaking in methanol, of the severed bases and apices of a small quantity of petals, caused removal of all visible coloration from both samples, as well as of the ultraviolet-absorbing material from the bases. A comparison of the absorption spectra of the basal and apical extracts (Fig. 2B) shows both to have the carotenoid absorption responsible for yellow coloration (maxima at 420, 443, and 470 nm), but to differ in the near ultraviolet, where the basal extract has a maximum (350 nm), and the apical extract a minimum (360 nm). Thin-layer chromatography (TLC) (on polyamide TLC-11) of the basal extract showed three components, absent from the apical extract. It was these components that we set out to identify.

A sample of whole petals (6 g, dry weight) was extracted by soaking them for 3 weeks in methanol. Evaporation of the extract gave 1.4 g of a dark residue, from which 0.4 g of carotenoid material was removed by ether extraction. The ether-insoluble material was chromatographed on 37 g of polyamide CC-6 with aqueous butanone. Virtually all of the absorption in the 340- to 380-nm region was accounted for by three chromatographic fractions, which corresponded to those found by TLC of the basal extract. The least polar fraction yielded 21 mg of pale yellow crystals (compound **1**) on crystallization from aqueous methanol. The fraction of intermediate polarity yielded 10 mg of a pale green material (compound **2**) on crystallization from a mixture of acetonitrile and methanol, and the most polar component produced a yellow amorphous solid (compound **3**) on at-