Enzymological Approaches to the Lipidoses

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ABSTRACT

There are now ten known heritable disorders of lipid metabolism for which the nature of the underlying enzymological defect is conclusively established. In addition to devising procedures for successful enzyme replacement therapy, much current work deals with the development of convenient, effective methods for the rapid diagnosis of patients with these disorders, the detection of heterozygous carriers of these diseases, and the monitoring of pregnancies at risk for these conditions. Clinical enzymologists are required to assume increasing responsibilities in the performance of these tests, and the present contribution describes the application of fundamental principles and discusses recent developments along this line. In particular, the development of facile chromogenic reagents for the diagnosis of patients with Niemann-Pick disease and Krabbe's disease is delineated. Previously, the diagnosis of such patients required the use of radioactivity labeled compounds and such testing was limited to relatively few research laboratories. The novelty of these reagents and their application comprise the major aspect of this presentation.

Introduction

The role and contributions of clinical enzymologists are becoming increasingly important for the management of hereditary metabolic diseases. The translation of basic research discoveries to practical benefits requires further extensive engagement of clinical scientists. Nowhere has this concept been more evident than in the control of the lipid storage diseases. Just over a decade ago, the pivotal advance in this area was provided through the elucidation of the enzymatic defect in Gaucher's disease. 12,15 It was specifically demonstrated for this disorder, and eventually for all of the nine known other lipid storage diseases, that these conditions are due to an insufficiency of hydrolytic enzymes required for the catabolism of the accumulating substances.

This discovery permitted the rapid development of sensitive, facile procedures for the diagnosis of patients with these conditions using readily available materials such as washed peripheral blood leukocytes 27 (figure 1), cultured skin fibroblasts 25 (figure 2) and, in some disorders, serum samples. 32 Initially, most of these tests required the use of radioactivi-
ity labeled lipids that accumulated in the various disorders. However, it soon became apparent that chromogenic or fluorogenic artificial substrates could be used in a number of these conditions.

Some very recent developments will be cited in this communication that now permit the detection of all except one of the known lipid storage diseases through the use of artificial substrates. These reagents have distinct advantages for day to day application in clinical chemistry laboratories where the lack of radioactive counting equipment precludes the utilization of labeled compounds. Therefore, the use of chromogenic and fluorogenic substances for the diagnosis of lipid storage diseases, the detection of heterozygous carriers of these disorders and the monitoring of pregnancies at risk for these conditions will be the main theme of this article. Only a minimum of clinical manifestations of these disorders will be cited since they are described extensively in previous contributions.5,6,7,9

Gaucher's Disease

This disorder and, in fact, all of the lipid storage diseases are characterized by the accumulation of derivatives of the long chain amino alcohol called sphingosine \[\text{CH}_3(\text{CH}_2)_{12}-\text{CH} = \text{CH} - \text{CH(OH)} - \text{CH(NH}_2) - \text{CH}_2\text{OH}\] to which a long chain fatty acid is linked via an amide bond to the nitrogen atom on carbon 2. This combination of sphingosine and fatty acid is called ceramide, and it is shared by all of the accumulating lipids. Various hexoses, oligosaccharides or phosphorylcholine are linked to carbon 1 of the sphingosine moiety of ceramide.
Thus, the lipid that accumulates in Gaucher’s disease is called glucocerebroside, and it consists of a single molecule of glucose joined by a β-glycosidic bond to ceramide. Glucocerebroside labeled in the glucose portion of the molecule with radiocarbon-14 was synthesized and this labeled compound was used to demonstrate the metabolic lesion in Gaucher’s disease. Here there is a deficiency of the enzyme that catalyzes the hydrolytic cleavage of the glucose portion of glucocerebroside according to Reaction 1.

1. Glucocerebroside + H₂O
   \[ \text{glucocerebroside-β-glucosidase} \]
   \[ \text{ceramide + glucose} \]

Radioactive glucocerebroside has been used extensively for the diagnosis of patients with Gaucher’s disease, the detection of carriers and the prenatal diagnosis of fetuses with this condition. The fluorescent substrate, 4-methylumbelliferyl-β-D-glucopyranoside (figure 3), has been used for a number of years to diagnose patients with Gaucher’s disease and the detection of carriers with extracts of cultured skin fibroblasts under rigidly specified conditions. An early attempt to use this fluorogenic compound for patient and carrier detection with leukocyte preparations has not provided the reliability required for general use. It has recently been found that this substrate may be used with confidence if leukocytes are homogenized and subjected to centrifugation. β-Glucosidase activity in the pellet obtained by this procedure is then determined with the fluorogenic glucoside. This procedure has been found to be reliable for diagnosing patients with Gaucher’s disease.

Recently, a chromogenic analogue of glucocerebroside (figure 4) has been used successfully for the diagnosis of

Gaucher’s disease. However, assays with this substrate are somewhat less sensitive than those with ¹⁴C-glucocerebroside or 4-methylumbelliferyl-β-D-glucopyranoside and may not be practical when leukocytes are used as the source of tissue to be analyzed.

Niemann-Pick Disease

The metabolic defect in Niemann-Pick disease was identified a decade ago as a deficiency of sphingomyelinase (Reaction 2).

2. Sphingomyelin + H₂O
   \[ \text{sphingomyelinase} \]
   \[ \text{ceramide + phosphorylcholine} \]

For nine of these years, the use of labeled sphingomyelin was required for
Figure 5. 2-Hexadecanoylamino-4-nitrophenylphosphorylcholine, a chromogenic analogue of sphingomyelin used for the diagnosis of patients with Niemann-Pick disease.

the diagnosis of patients and the detection of carriers of Niemann-Pick disease. In 1975, a major diagnostic breakthrough occurred through the synthesis of a useful chromogenic analogue of sphingomyelin that has proven to be completely reliable for the enzymatic detection of Niemann-Pick disease and the carriers of this trait and the monitoring of pregnancies at risk for this disorder (figure 5). This compound may be used for the diagnosis of Niemann-Pick disease using tissue specimens or extracts of cultured skin fibroblasts as source of enzyme. At the moment, radioactive sphingomyelin is preferred when leukocytes are used since it provides a slightly more sensitive test system than the chromogenic analogue.

Krabbe's Disease

Here there is a deficiency of the enzyme that catalyzes the cleavage of the molecule of galactose from galactocerebroside (Reaction 3).

3. Galactocerebroside + H₂O
   galactocerebroside-β-galactosidase
   ceramide + galactose

Until recently, the use of radioactive galactocerebroside was mandatory for the diagnosis of patients and the detection of carriers of this disorder. However, a chromogenic analogue has been synthesized of galactocerebroside similar to that used for the diagnosis of Gaucher's disease and Niemann-Pick disease except that galactose is linked by a β-glycosidic bond to 2-N-hexadecanoylamino-4-nitrophenol instead of glucose or phosphorylcholine. This galactoside derivative has been found to be useful for the diagnosis of patients with Krabbe's disease using samples of brain or liver tissue obtained from patients with this metabolic disorder.

Assay conditions have now been established which permit the use of the galactocerebroside analogue for the diagnosis of patients and carriers of Krabbe's disease using extracts of cultured skin fibroblasts.

Metachromatic Leukodystrophy

Patients with this hereditary disorder lack arylsulfatase A activity in their organs and tissues. This enzyme catalyzes the cleavage of sulfuric acid from sulfatide, the 3-O'-sulfate ester of galactocerebroside (Reaction 4).

4. Sulfatide + H₂O
   sulfatidase (aryl sulfatase A)
   galactocerebroside + H₂SO₄

Even before this defect had been demonstrated with the natural lipid substrate, Austin and co-workers had clearly shown that there was a decrease in sulfatase activity in tissues of patients with this disorder through the use of nitrocatechol sulfate as substrate. These observations have stood the test of time, and this chromogenic substrate is still widely used for the diagnosis of patients with metachromatic leukodystrophy and the detection of heterozygotes.
Fabry's Disease

In contrast with all of the other known lipid storage diseases which are autosomal recessive disorders, Fabry's disease is transmitted as an X-chromosome linked recessive trait. In this disorder, ceramidetrihexoside accumulates because of an insufficiency of the galactosidase that catalyzes the cleavage of the terminal molecule of galactose from ceramidetrihexoside\(^1\) (Reaction 5).

\[
\begin{align*}
5. & \quad \text{Ceramide-glucose-galactose-galactose + H}_2\text{O} \\
& \quad \text{ceramidetrihexoside-} \alpha- \text{galactosidase} \\
& \quad \text{ceramide-glucose-galactose + galactose}
\end{align*}
\]

\(^3\)H-labeled ceramidetrihexoside has been used to detect patients and carriers of this disorder. However, most current diagnostic work is based on the demonstration by Kint that patients with Fabry's disease have decreased \(\alpha\)-galactosidase activity in their tissues.\(^2\) Either 4-methylumbelliferyl-\(\beta\)-D-galactopyranoside or p-nitrophenyl-\(\alpha\)-D-galactopyranoside may be used for this assay. Heterozygotes and afflicted fetuses may readily be detected with these substrates.\(^17\)

Tay-Sachs Disease

This inherited metabolic disorder is caused by a deficiency of the enzyme that catalyzes the cleavage of the molecule of N-acetylgalactosamine from the acidic glycolipid called Tay-Sachs ganglioside\(^2\) (Reaction 6).

\[
\begin{align*}
6. & \quad \text{Ceramide-glucose-galactose-} \\
& \quad \text{(N-acetylneuraminic acid)-N-} \\
& \quad \text{acetylgalactosamine + H}_2\text{O} \\
& \quad \text{hexosaminidase} \\
& \quad \text{ceramide-glucose-galactose-} \\
& \quad \text{N-acetylneuraminic acid + N-acetylgalactosamine}
\end{align*}
\]

Patients with this disorder may be readily diagnosed by measuring hexosaminidase activity in tissues and blood with the fluorogenic substrate 4-methylumbelliferyl-\(\beta\)-D-N-acetylglucosaminide.\(^3\)\(^2\),\(^3\)\(^5\) There are two major hexosaminidase isozymes in mammalian cells\(^3\)\(^9\) and only one of them, called Hex-A, is lacking in most patients with Tay-Sachs disease. The activity of the other hexosaminidase isozyme, called Hex-B, is greatly increased. Therefore, it is necessary to differentiate between these activities in order to carry out assays for homozygotes and heterozygous carriers of Tay-Sachs disease.

The most widely used procedure for this differentiation is the selective heat inactivation of Hex-A.\(^8\),\(^3\)\(^2\),\(^3\)\(^5\) Total tissue hexosaminidase activity is measured in an aliquot of the sample under investigation. Another aliquot is heated for four hours at 50\(^\circ\)C. Hex A activity is destroyed by this procedure and the residual hexosaminidase activity represents the Hex B component. The amount of Hex A activity is determined by subtracting Hex B activity from total hexosaminidase activity. When performed carefully, this test permits reliable identification of patients with Tay-Sachs disease and the detection of carriers. Other techniques for determining Hex A and Hex B activity include separation of the isozymes by gel electrophoresis,\(^3\)\(^9\) inactivation of Hex A under acidic conditions\(^3\)\(^7\) and separation of isozymes by small ion-exchange columns.\(^1\)\(^9\)

In contrast with most Tay-Sachs patients, some individuals, currently referred to as patients with Sandhoff's disease, have very little total hexosaminidase activity in their tissues.\(^4\)\(^0\) The diagnosis of these patients and the detection of carriers is also feasible using 4-methylumbelliferyl-\(\beta\)-D-N-acetylglucosaminide as substrate.\(^5\)
Clinical chemists should be aware of two other aspects of Tay-Sachs disease which might prove troublesome. A few patients with classic manifestations of Tay-Sachs disease have been described where Hex A and B activities are perfectly normal when measured with artificial substrates such as 4-methylumbelliferyl-β-D-N-acetylglucosaminide or p-nitrophényl - β-D - N - acetylgalactosaminide. In contrast, the catabolism of the ganglioside itself is severely compromised.

Furthermore, it has recently been discovered that there are perfectly normal individuals without detectable Hex A activity in their tissues who, in fact, can catabolize Tay-Sachs ganglioside. If one were to use a chromogenic or fluorogenic substrate for prenatal detection as commonly practiced at the present time, such a fetus would be classified as a Tay-Sachs homozygote. Here again, one must rely on the use of Tay-Sachs ganglioside specifically labeled in the N-acetylgalactosaminyl portion of the molecule for correct diagnosis. Procedures for the preparation of this substrate have been published.

Generalized Gangliosidosis

This disorder is caused by a lack of the enzyme that catalyzes the cleavage of the terminal molecule of galactose from ganglioside G₄₁₃₄ (Reaction 7).

7. Ceramide-glucose-galactose-(N-acetylneuraminic acid)-N-acetylglactosamine-galactose (G₄₁₃₄) + H₂O

| β-galactosidase |
|-----------------
| ceramide-glucose-galactose-(N-acetylneuraminic acid)-N-acetylglactosamine + galactose |

Patients and carriers may be readily identified by measuring tissue β-galactosidase activity with 4-methylumbelliferyl-β-D-galactopyranoside or p-nitrophényl-β-D-galactopyranoside. The prenatal diagnosis of fetuses with this condition has also been demonstrated through the use of these substrates.

Fucosidosis

Patients with this disorder have a coarseness of the skin, bony changes, organomegaly and mental retardation. Fucose-containing H-isoantigenic lipids and a dekasaccharide accumulate in various tissues of these patients because of a lack of α-fucosidase activity. This enzymatic defect is readily detected using 4-methylumbelliferyl-α-L-fucopyranoside as substrate.

Farber's Disease

Patients with this rare inherited disorder of disseminated lipogranulomatosis have hoarseness of the voice, a brownish dermatitis and mental retardation. Fucose-containing H-isoantigenic lipids and a dekasaccharide accumulate in various tissues of these patients because of a lack of α-fucosidase activity. This enzymatic defect is readily detected using 4-methylumbelliferyl-α-L-fucopyranoside as substrate.

Conclusion

It is apparent that clinical chemists will be required to become increasingly involved in the use of enzyme assays for the diagnosis of heritable metabolic disorders, the detection of symptom-free heterozygous carriers of these deleterious traits and the monitoring of pregnancies at risk for these conditions. There is a
clear movement towards the rapid application of these tests to medical practice. Since weighty decisions are based on the results of these determinations, it is imperative that the clinical scientist familiarize himself with the concepts and techniques used in these analyses. At the present time, considerations involving the care with which these tests are performed and procedures for quality control of these assays are receiving intensive examination.

References


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