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Biotinidase Deficiency: A Novel Vitamin Recycling Defect

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The recent finding that biotinidase deficiency is the primary biochemical defect in late-onset multiple carboxylase deficiency has stimulated new interest in the inherited disorders of biotin-dependent carboxylases. The clinical and biochemical features of biotinidase deficiency are discussed. We also speculate about two exciting areas currently being investigated: the localization of action of biotinidase, and the possible role of the enzyme as a binding or carrier protein for biotin.

Multiple carboxylase deficiency (MCD) (McKusick 25327) describes a group of inherited metabolic disorders which are characterized by deficient activities of the biotin-dependent carboxylases (Sweetman, 1981; Wolf and Feldman, 1982). Based on differences in clinical features and the times of their appearance, MCD has been catagorized into two major forms. Most children with the first form, neonatal or early-onset MCD, have been shown to have a primary enzyme deficiency in the activity of biotin holocarboxylase synthetase, the enzyme that attaches biotin covalently to the various apocarboxylases forming holoenzyme (Burri *et al.*, 1981; Saunders *et al.*, 1982). We have recently demonstrated that most individuals with the second form, juvenile or late-onset MCD, are deficient in the activity of biotinidase [EC 3.5.1.12] (Wolf *et al.*, 1983a,b). This enzyme catalyzes the cleavage of biotin from biocytin (ε-*N*-biotinyl-L-lysine) or biotinyl-peptides (Craft, 1984), the products of carboxylase degradation (Wright *et al.*, 1954; Thoma and Peterson, 1954). Biotinidase is not only important for the recycling of endogeneous biotin but it appears to play an integral role in the processing of dietary, protein-bound biotin. This disorder is unique among the inherited vitamin-responsive metabolic diseases for two reasons. First, all children with biotinidase deficiency have improved clinically following the administration of oral biotin and treatment initiated early has been essentially "curative". Second, we now have evidence that biotinidase deficiency can be successfully treated with daily doses of biotin in the physiologic range rather than with pharmacologic doses.

THE ENZYME DEFECT

Biotinidase activity has been determined by measuring the release of biotin from biocytin, using several microbiological assays (Wright *et al.*, 1954; Koivusalo and Pispa, 1963) and by the release of chromophoric amino compounds from biotinylated substrates (Knappe *et al.*, 1963; Pispa, 1965). We determined enzyme activity using a modification of the method of Knappe *et al.* (1963), in which *p*-aminobenzoate (PABA) is released from the substrate, *N*-biotinyl-*p*-amino-benzoate.

The biotinidase activity in the serum of 18 healthy, fasting, normal children and adults was 5.80 ± 0.89 nmol min^{-1} ml^{-1} (Table 1). Biotinidase activity in the serum of children with the clinical features of late-onset MCD was deficient with a mean of 3.4% of mean normal activity. No increase in activity was found after increasing the concentration of substrate in the assay to 60 $mmol^{-1}$ (an 800-fold increase). The activities in the serum of parents who were available for study were intermediate between those of the affected children and normal controls with a mean of 57% of mean control activity. Biotinidase activity in serum from one patient with biotin holocarboxylase synthetase deficiency was normal (Sweetman and Burri, 1981). We demonstrated that the deficient activity in the serum of affected children was not due to the presence of inhibitors, including biotin at concentrations usually attained after treatment with pharmacologic doses of the vitamin.

Very little biotinidase activity was detectable in concentrated extracts of fibroblasts from normal individuals using the colorimetric assay (Wolf *et al.*, 1983a). Therefore we developed a sensitive radioassay based on the liberation of [^{14}C-carboxyl]-*p*-amino-benzoate from *N*-biotinyl-[^{14}C-carboxyl]-*p*-amino-benzoate (Wolf and Secor McVoy, 1984). This assay is approximately 100 times more sensitive than the colorimetric method.

Using the radioassay we found biotinidase activity in extracts of peripheral blood leukocytes and fibroblasts of normal individuals but essentially no detectable activity in the serum of individuals who were shown previously to be biotinidase-deficient using the colorimetric assay (Wolf *et al.*, 1983a). Activities in the extracts of peripheral blood leukocytes and fibroblasts of patients with biotinidase-deficient serum were found to be less than 1% of mean normal activities. The specific activities in normal leukocytes and fibroblasts are similar to those reported in other mammalian tissues (Koivusalo and Pispa, 1963; Pispa, 1965). These studies and a report of deficient activity in the liver of an affected child (Gaudry *et al.*, 1983) also demonstrate that the deficiency of biotinidase activity in affected patients is not confined to serum and substantiate further that biotinidase deficiency is the primary defect in most patients with late-onset MCD.

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Table 1 Biotinidase activity in the sera of affected children and their parents

Group	*Number of samples*	*Biotinidase activity in serum* (nmol PABA $min^{-1} ml^{-1}$) *Mean**	*Range*
Normal individuals	18	5.80 ± 0.89	4.30–7.54
Affected children	15	0.20	0–0.90
Parents	18	3.29 ± 0.60	2.40–4.50

* Duplicate determinations were performed on each sample. Values are means ± 1 SD

CLINICAL AND BIOCHEMICAL FEATURES

Fifteen children with biotinidase deficiency have been diagnosed by our laboratory. The clinical features of these and five other patients with biotinidase deficiency (Charles *et al.*, 1979; Leonard *et al.*, 1981, case 4; Munnich *et al.*, 1981b; Gaudry *et al.*, 1983) are summarized in Table 2. There are 12 females and eight males from 16 families; consanguinity of the parents was reported in four families. This, together with the finding of about half-normal activity in the parents, indicates that biotinidase deficiency is inherited as an autosomal recessive trait. All of the children exhibited some or all of the symptoms usually seen in patients with late-onset MCD (Sweetman, 1981; Bonjour, 1981; Wolf and Feldman, 1982). The age of onset of symptoms varied from 3 weeks to 2 years of age (median age is 3 months, mean age is 6.3 months). The disorder was usually suspected when the children exhibited alopecia, skin rash and seizures, with the seizures frequently being the initial symptom. Fourteen of the patients were diagnosed because they had organic aciduria in addition to those neurologic and cutaneous features characteristic of the late-onset disorder. Of the remaining patients some had either the neurologic features or the cutaneous features characteristic of MCD; some had both but did not exhibit metabolic acidosis or organic aciduria. There is clinical variability among affected individuals from different families and, as demonstrated by siblings with the disorder, there is also considerable variability in expression of the disorder among affected family members (Wolf *et al.*, 1983b).

Table 2 Clinical and biochemical features of biotinidase deficiency

Feature	*Frequency*
Alopecia	17/20
Skin rash	15/20
Seizures	14/20
Ataxia	9/20
Conjunctivitis	9/20
Hypotonia	9/20
Hearing loss	8/16
Developmental delay	10/18
Fungal infections	6/20
Metabolic acidosis	14/19
Lactic acidosis	13/18
Hyperammonaemia	6/13
Organic acidaemia	14/18

In previously reported cases the diagnosis of the late-onset disease depended in part on the presence of demonstrable organic aciduria. Failure to detect this finding would have excluded about one-third of our cases of biotinidase deficiency. Although the biochemical abnormalities attributed to biotinidase deficiency are often life-threatening, they appear to represent relatively late effects of the disorder. The cutaneous symptoms and some of the neurologic signs are similar to those seen in biotin deficiency states (Sweetman *et al.*, 1981; Mock *et al.*, 1981; McClain *et al.*, 1982) and they usually occur early in the course of the disease. Biotin deficiency does not alter biotinidase activity. The enzyme activity in the sera of several patients who became biotin-deficient while receiving parenteral hyperalimentation was normal (Kien *et al.*, 1981) and biotin-deficient rats have similar biotinidase activities to rats receiving adequate dietary biotin (Suchy *et al.*, 1984). It seems likely that the cutaneous and neurologic symptoms of biotinidase deficiency result from a mild to moderate depletion of biotin when the residual carboxylase activities are still adequate to maintain normal metabolic balance. Only after protracted biotin deficiency do ketoacidosis and organic aciduria appear.

Hearing loss, which in several children was attributable to neurosensory impairment, has been diagnosed in about half the children with biotinidase deficiency (Taitz *et al.*, 1983; Wolf *et al.*, 1983c). Patients usually exhibited hearing loss before the initiation of biotin treatment but the deficit did not appear to improve after biotin therapy. It is possible that the hearing loss is caused by the accumulation of organic acids or by the accumulation of biocytin or larger biotinyl-peptides which, in the biotin-deficient state, may alter the metabolic pathways involved in the development and/or function of the auditory system (Taitz *et al.*, 1983). Although these metabolites should continue to accumulate after biotin treatment is begun, further hearing loss may be prevented in the presence of adequate biotin.

Immunoregulatory dysfunction has been reported in several children with biotinidase deficiency (Cowan *et al.*, 1979; Fischer *et al.*, 1982). However, too few patients have undergone immunological evaluation to enable a clear description of either the specific immunological abnormalities in biotinidase deficiency, or their clinical significance.

Because of their inability to recycle biotin, biotinidase-deficient children are dependent upon exogenous biotin to prevent the clinical and biochemical features of biotin deficiency. Previous reports have described 'impaired' intestinal absorption of biotin in two patients with MCD (Munnich *et al.*, 1981a; Thoene *et al.*, 1983). However, both patients have since been shown to be biotinidase-deficient (Thoene and Wolf, 1983; J.M. Saudubray, personal communication). It has been shown in one of these patients that the response to oral biotin is normal if the loading test is conducted when the tissues are not depleted of biotin (Thoene and Wolf, 1983). Loading tests, performed on patients whose tissues are severely biotin-depleted, apparently result in the rapid entry of the vitamin into these tissues and in plasma concentrations of biotin which are misleadingly low. We would expect that the second patient would respond similarly if the appropriate loading study was performed.

Humans cannot synthesize biotin and therefore must derive the vitamin from the turnover of biotin-containing enzymes and from the absorption of biotin from dietary and/or microbial origin. The concentrations of free and protein-bound biotin in foods are variable but most of the biotin in foods such as meats and cereals is protein-bound (György, 1939; Thompson *et al.*, 1941). Therefore biotinidase may also play a critical role in the processing of dietary, protein-bound biotin. We have shown that although intestinal mucosa has biotinidase activity, the activity is not enriched in intestinal brush-border membranes (Wolf *et al.*, 1984). Biotinidase may be secreted by the columnar or specialized glandular cells. We found no activity in bile but considerable activity in pancreatic juice. The combined action of secreted gastrointestinal proteases and those associated with brush-border membranes on dietary, biotin-containing proteins may result in the liberation of biocytin or biotinyl-peptides. These compounds may then either be absorbed and hydrolyzed in the mucosa or be hydrolyzed in the intestinal lumen by biotinidase which originates from bacteria, pancreatic juice, the intestinal mucosa or all of these sources. Furthermore, if biotinidase production by intestinal flora is quantitatively unimportant, then patients with biotinidase deficiency would lack a mechanism for liberating protein-bound biotin from food and, if the contribution of the microflora to the free biotin pool is small or negligible, then these patients would depend entirely on dietary free biotin to meet their requirements for the vitamin.

Children with biotinidase deficiency have been treated successfully with pharmacologic doses of biotin, 10–20 mg day^{-1}, an empirically determined dose rate. Patients with holocarboxylase synthetase deficiency must be treated with pharmacologic doses of biotin and some are unable to normalize their carboxylase activities even with biotin dosages as high as 80 mg day^{-1}. This phenomenon is attributable to an extremely high K_m for biotin in these patients (Sweetman and Burri, 1981). In contrast, all patients with biotinidase deficiency have improved following treatment with pharmacologic doses of biotin. It has been suspected that these doses supply more biotin than is actually required to meet the metabolic needs of these patients. One patient who was brought to our attention recently has been asymptomatic for eight years on a daily dose of biotin of about 150 μg day^{-1} (Diamantopoulos *et al.*, 1985). An important modifying factor in the treatment of this disorder may be the amount of free biotin in the diet; thus individuals consuming diets containing predominantly free biotin may require less supplemental biotin that those whose diet consists mostly of biotin in the bound form.

PRENATAL DIAGNOSIS AND NEONATAL SCREENING OF BIOTINIDASE DEFICIENCY

Using the radioassay we have found that biotinidase activity is also measurable in both amniotic fluid and cultured amniotic cells of normal pregnancies obtained by amniocentesis (Secor McVoy *et al.*, 1985). Since enzymes in amniotic fluid may be of maternal or fetal origin, the definitive diagnosis of biotinidase deficiency in the fetus depends on the determination of enzyme activity in extracts of amniotic fluid cells.

Although the tissues of heterozygous mothers have half-normal biotinidase activity, affected neonates are asymptomatic. This indicates that these mothers can supply the developing infant with adequate free biotin. However, the prenatal diagnosis of biotinidase deficiency may indicate whether prenatal treatment with biotin is warranted.

We have also developed a method of neonatal screening for biotinidase deficiency which involves the qualitative colorimetric assessment of biotinidase activity in the same samples of whole blood spotted on filter paper used in phenylketonuria screening (Heard *et al.*, 1984a). Samples with biotinidase activity show a characteristic purple color after incubation with *N*-biotinyl-*p*-aminobenzoate, whereas those with little or no activity remain straw-colored. Positive screening tests can be confirmed by a quantitative assay of enzyme activity using additional samples of dried blood or fresh serum. A pilot study, using samples obtained by the Commonwealth of Virginia for phenylketonuria testing, is currently being conducted to estimate the incidence of the disorder.

This technique also offers a simple, rapid method for the physician to obtain specimens from patients suspected of having biotinidase deficiency. Since the enzyme activity in the blood spots is stable for up to 18 months the cards may be sent to an appropriate reference laboratory.

SPECULATION

There are several major questions about the function of biotinidase that remain unanswered. First, where does the enzyme primarily function? Our preliminary studies have shown that serum biotinidase activity correlates positively with the concentration of serum albumin. The concentrations of albumin and the activities of biotinidase in sera of patients with cirrhosis were lowered (Weiner *et al.*, 1983), indicating that in the

human as well as in the rat (Pispa, 1965) serum biotinidase originates principally from the liver. Given that the pH optimum for biotinidase activity is pH 5–7.5 (Pispa, 1965, Wolf *et al.*, 1983a) and that there is evidence that at least pyruvate carboxylase is degraded in lysosomes (Chandler and Ballard, 1983) it is attractive to assume that biotinidase is localized in the lysosome where it can readily hydrolyze biotinyl-substrates. However, subcellular fractionation studies show that biotinidase activity is enriched in the microsomal fraction (Pispa, 1965; Heard *et al.*, 1985), and not enriched in the lysosomal fraction. The enzyme, which migrates in the α_1-region on serum electrophoresis, is sialylated in serum and is asialylated in tissues (Heard *et al.*, 1985). In addition, biotinidase activity is readily determined in various tissues with secretory function, such as liver, fibroblasts, leukocytes and pancreas (Pispa, 1965; Wolf and Secor McVoy, 1984), as well as in pancreatic juice and isolated zymogen granules (Heard *et al.*, 1984b). These results are compatable with a model in which biotinidase is a secretory enzyme that hydrolyzes the products of carboxylase degradation that reach the blood. Finding a relatively greater specific activity of biotinidase in the serum (about 120 pmol min^{-1} mg $protein^{-1}$) than in the tissues (10–50 pmol min^{-1} mg $protein^{-1}$) could be explained if the enzyme primarily functioned in the serum. In fact, there is evidence that biotin originating from degraded carboxylases is not recycled in the cells, but in the extracellular compartment (Chandler and Ballard, 1983; Freytag and Utter, 1983).

Second, is biotinidase a binding or carrier protein for biotin in serum? The specificity of biotinidase resides in the biotinyl moiety of its substrate. If few molecules of biocytin or biotinyl-peptide are available to interact with biotinidase in the serum at any instant, then biotin which is a known competitive inhibitor of biocytin for biotinidase should be in equilibrium with the enzyme. Biotinidase has been purified 5000-fold to homogeneity from serum and has a molecular weight of approximately 76 000 daltons (Craft and Goss, 1982). Therefore, we would expect there to be about 200 pmol enzyme ml $serum^{-1}$ and, if a typical serum biotin concentration of 2 pmol ml^{-1} is assumed, then the ratio of enzyme to biotin would be 100:1. Baumgartner *et al.* (1982) attributed abnormally high urinary output of biotin by a biotinidase-deficient patient to defective tubular reabsorption but also suggested that it could be due to the absence of or to a functionally deficient plasma biotin-binding protein. Biotinidase activity is present in the kidney but it is not enriched in the renal brush border membrane (Wolf *et al.*, 1984) and probably does not play a role in biotin recycling in the kidney.

If biotinidase is a biotin-binding protein in serum and is absent from the sera of biotinidase-deficient patients or so altered structurally as to be unable to interact with biotin, then the increased excretion of biotin in these patients can easily be explained. Assuming that in the biotin-replete state normal individuals and biotinidase-deficient patients have similar concentrations of biotin in their sera, then the gradient between the serum and the lumen of renal tubules for free biotin would be greater in the patients and, hence, they would excrete more biotin. Figure 1 summarizes schematically the various possible mechanisms of biotinidase function. Answers to these important questions are currently being actively pursued in our laboratory.

CONCLUSION

Biotinidase is necessary for the normal recycling of biotin that has been incorporated into the carboxylases. The presence of biotinidase activity in normal individuals confers some independence from exogenous biotin. In patients with biotinidase deficiency, the biotin salvage pathway is blocked and sufficient biotin must be ingested to prevent the development of symptoms of biotin deficiency from occurring. The potential exists for prenatal and neonatal diagnosis of the disorder, and we have initiated a pilot neonatal screening program to estimate its incidence. Daily physiologic doses, rather than pharmacologic doses, of biotin are likely to be sufficient to alleviate the symptoms of biotin deficiency, and biotinidase deficiency thus joins pyridoxine-responsive seizures as a specific and treatable form of infantile seizures. Elucidation of biotinidase deficiency as the primary defect in late-onset MCD has not only furthered interest in the inherited disorders of biotin-dependent carboxylases, but has led to new approaches for understanding the basic metabolism of biotin and its role in nutrition.

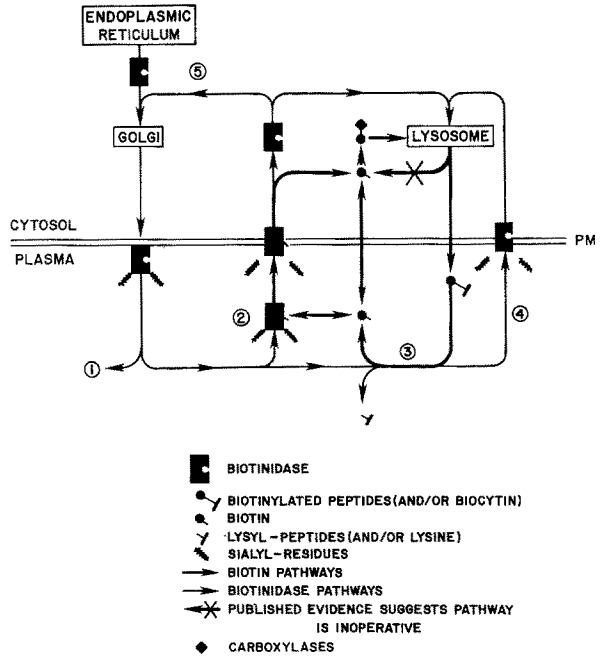

Figure 1 A schematic model for the recycling of biotin and biotinidase. The pathways labeled in the diagram represent the following processes: (1) transfer of biotinidase to tissues other than the sites of production; (2) binding of biotin to biotinidase (or another biotin-binding protein) and transport of this bound biotin into cells; (3) cleavage of biotinylated peptides or biocytin; (4) desialylation and re-entry of biotinidase into cells which may be followed by degradation; or (5) recycling of the enzyme.

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