Research article

Increased urinary excretion of a 3-(3-hydroxyphenyl)-3-hydroxypropionic acid (HPHPA), an abnormal phenylalanine metabolite of *Clostridia* spp. in the gastrointestinal tract, in urine samples from patients with autism and schizophrenia

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A compound identified as 3-(3-hydroxyphenyl)-3-hydroxypropionic acid (HPHPA) was found in higher concentrations in urine samples of children with autism compared to age and sex appropriate controls and in an adult with recurrent diarrhea due to *Clostridium difficile* infections. The highest value measured in urine samples was 7500 mmol/mol creatinine, a value 300 times the median normal adult value, in a patient with acute schizophrenia during an acute psychotic episode. The psychosis remitted after treatment with oral vancomycin with a concomitant marked decrease in HPHPA. The source of this compound appears to be multiple species of anaerobic bacteria of the *Clostridium* genus. The significance of this compound is that it is a probable metabolite of *m*-tyrosine (3-hydroxyphenylalanine), a tyrosine analog which depletes brain catecholamines and causes symptoms of autism (stereotypical behavior, hyperactivity, and hyperreactivity) in experimental animals.

Keywords: *m*-tyrosine, 3-hydroxyphenylalanine, metronidazole, enkephalins, Lactobacillus acidophilus

Introduction

For the past 10 years, I have evaluated by gas-chromatography mass-spectrometry biochemical abnormalities that appear to be of microbial origin in urine samples of children with autism and other developmental disorders as well as adults with a wide variety of disorders, after the discovery that certain putative microbial metabolites appeared in higher than normal values in urine samples of two brothers with autism.[1] These findings were of especial interest to me because of a report that autistic children have a greater incidence of ear infections than age-matched peers; that lower functioning autistic children had an earlier onset of ear infections than their higher functioning autistic peers; and that the ears of children with autism were anatomically positioned differently than those of normal children, perhaps leading to greater ear infection susceptibility.[2] Intestinal overgrowth of yeast and anaerobic bacteria are well-documented sequelae of the common oral antibiotics used to treat ear infections.[3–6] Therefore, it is possible that abnormally elevated biochemical products of

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abnormal micro-organisms in the gastrointestinal tract may play a role in the etiology of autism just as abnormal elevations of phenylalanine and its metabolites cause the disorder phenylketonuria (PKU). During testing, an unusual compound was detected in high concentrations in samples from children with autism, child psychosis, attention deficit hyperactivity, and in adults with severe depression, seizures, or schizophrenia. Since this compound in urine has not been adequately characterized, I began an intense investigation to identify it and determine its source.

Subjects and methods

Low-resolution electron impact gas-chromatography/mass spectrometry was performed as previously described.[1] In order to identify unknown compounds, the same procedure was performed except that perdeuterated *N,O-bis*(trimethylsilyl) acetamide (BSTFA) was used in place of non-deuterated BSTFA. High resolution gas-chromatography/mass spectrometry was performed on a VG 70-250S double focusing mass spectrometer interfaced with a Hewlett Packard model 5890 gas chromatograph. The column used was a 15-m, DB-1 capillary column with a 0.25 mm internal diameter and a 1.0 micron film from J & W Scientific (Folsom, CA, USA). The injection mode was splitless, and the injection volume was 0.5 μl. Initial mass calibration was performed using perfluorinated kerosene (PFK) over the mass range of 35–650 Da. PFK was also used over a limited mass range to calibrate for the high-resolution mass measurements. Once the compound of interest was located by low-resolution analysis, the resolution of the instrument was increased to 10,000 (10% valley criterion). The theoretical mass for 3-(3-hydroxyphenyl)-3-hydroxypropionic-TMS3 is 398.1765 Da. Two PFK peaks of known accurate mass (392.9760 and 404.9760 Da), which bracket the expected mass of the unknown, were located. The mass spectrometer was set to scan only this limited mass range. The sample was then injected and, at the proper retention time, the data acquisition system was started. 3,4-Dihydroxyphenylpropionic acid and 3-(4-hydroxyphenyl)-2-hydroxypropionic acid were obtained from Sigma Chemical Company (St Louis, MO, USA). Other common names for 3,4-dihydroxyphenyl-propionic acid are hydrocaffeic acid and 3,4-dehydroxy-hydrocinnamic. The minimum purity claimed by the manufacturers was 98%. Both Sigma and Fluka provided NMR analyses on the lots of materials to confirm the isomeric composition.

For quantitative analysis of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid, the response factor for 3,4-dihydroxyphenylpropionic acid was used as a surrogate calibration standard for the 3-(3-hydroxyphenyl)-3-hydroxypropionic acid using the reconstructed ion chromatograph signal of the ion at *m/z* 398 for quantitation. Undecanoic acid was used as the internal standard as described previously.[1] All results were normalized to urine creatinine as a way of minimizing variability due to differences in fluid intake.

Urine samples were obtained from both in-patients and out-patients at a pediatric hospital as well as from physicians submitting samples for my reference laboratory service. Urine samples (14 from males and 14 from females) were obtained from babies under 2 months old at a well-baby clinic at a local pediatric hospital. Urine samples from normal control children, 30 of each sex between the ages of 2–13 years, were obtained from children of local hospital employees. Normal adult values (n = 19) were obtained from adult volunteers (11 females and 8 males). Urine samples were obtained from children with autism between the ages of 2–13 years (211 males and 51 females). The ratio of male-to-female samples reflects the approximate male-to-female ratio of autism incidence in the population. The autistic children were either out-patients at the hospital or were referred for testing at the hospital. We requested first morning urine samples but did not verify compliance with this request. Baby urine samples were collected into tape-on urine collection bags during the night. Pediatric neurologists, developmental pediatricians, or child psychiatrists, using DSM-IV criteria, had made the diagnosis of autism. Dr Walter Gattaz at the Central Mental Health Institute at Mannheim, Germany collected samples from 12 drug-free patients with schizophrenia (4 males and 8 females). An additional sample from a drug-free patient with first onset of schizophrenia with auditory hallucinations was submitted by the attending physician. Urine samples were randomly collected in plastic screw-cap containers and stored at –20ªC until tested. The procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 1983.

Results

A typical urine chromatogram from a child with autism is shown in Figure 1A. A large peak from the TMS derivatives of urine extracts of patients with autism, all 12 drug-free patients with schizophrenia, and one patient with child psychosis and eluting shortly after the citric acid trimethylsilyl (TMS)

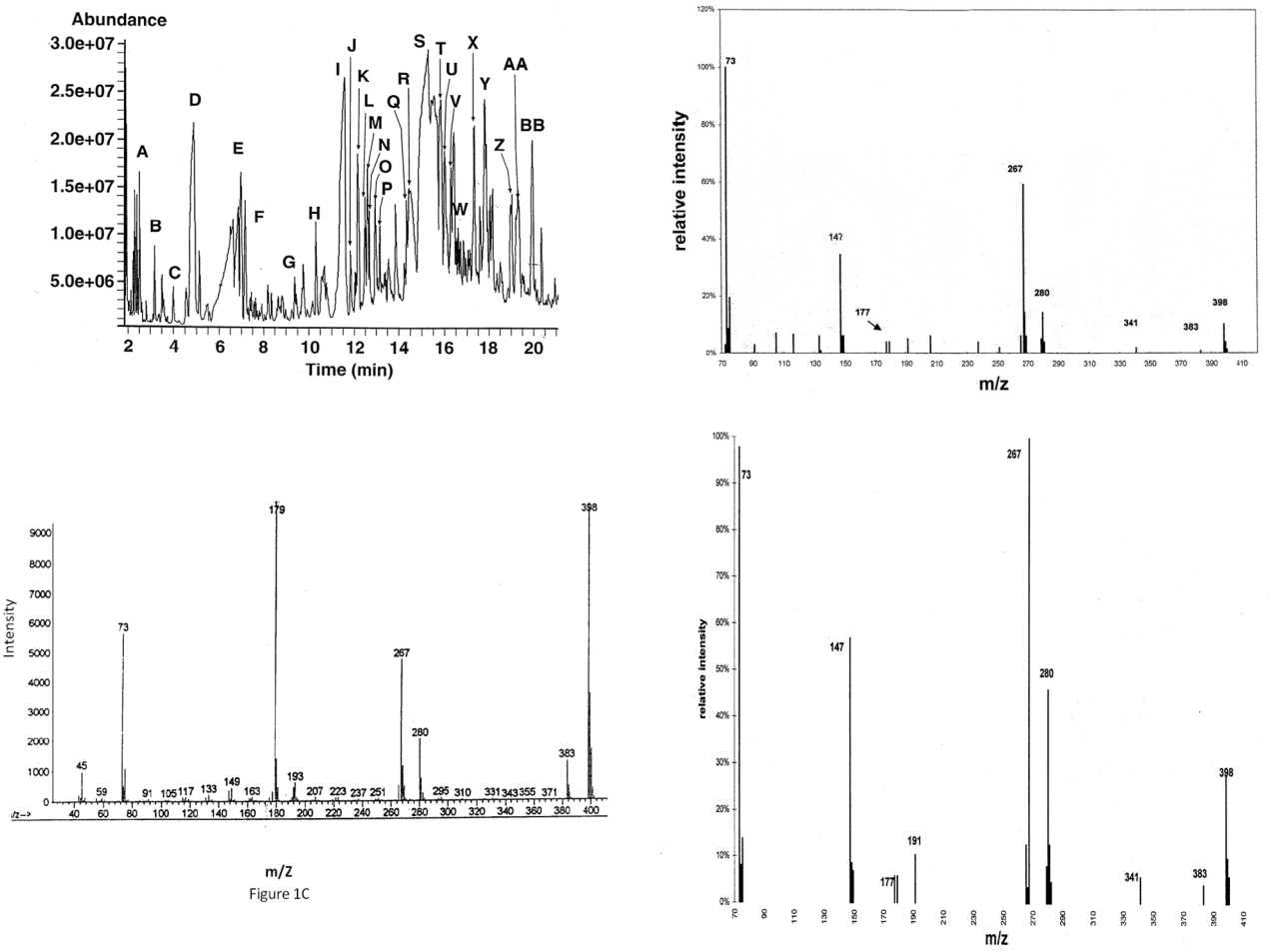

Figure 1 (A) Total ion current GC/MS chromatogram of the derivatized urine extract of child with autism. Peaks are identified as follows: A, glycolic; B, oxalic; C, 3-hydroxyisobutyric; D, urea; E, phosphoric; F, succinic; G, deoxytetronic; H, citramalic; I, undecanoic (internal standard); J, unidentified; K, 3-hydroxyphenylacetic; L, 2-oxoglutaric; M, 4-hydroxyphenylacetic; N, furandicarboxylic; O, furancarbonylglycine; P, tartaric; Q, arabinose; R, aconitic; S, hippuric; T, citric; U, 3-(3-hydroxyphenyl)-3-hydroxypropionic acid; V, vanillylmandelic; W, 3-indoleacetic; X, ascorbic; Y, citric analog; Z, uric; AA, unidentified; BB, hydroxyhippuric. Modified from Shaw W et al Clin Chem 41:1094-1104,1995 with permission. (B) Electron-impact mass spectrum of unknown compound in the urine sample extract of a child with autism. (C) Electron-impact mass spectrum of 3,4-dihydroxyphenylpropionic acid TMS derivative. (D) Electron-impact mass spectrum of authentic 3-(3-hydroxyphenyl)-3-hydroxypropionic acid TMS derivative.

derivative has an electron-impact mass spectrum with prominent ions at *m/z* 73,147,267, 280, 341,383, and 398. The mass spectrum in Figure 1B was from a child with autism. This mass spectrum is suggestive of a dihydroxy-substituted phenylpropionic acid tri-TMS derivative. The mass spectrum of 3,4-dihydroxyphenylpropionic acid tri (TMS) derivative (Fig. 1C) is very similar to the compound in urine but has a very intense ion at *m/z* 179, which is weak in the urine compound. Small quantities (< 1 mmol/mol creatinine) of 3,4 dihydroxyphenylpropionic acid derivative were sometimes found in a variety of urine samples. Furthermore, the major isomer in urine does not match the retention time of commercial 3,4-dihydroxyphenylpropionic acid tri (TMS) derivative. None of the published mass spectra of all of the other isomers of dihydroxyphenylpropionic acid tri (TMS) derivatives[7] or that of 3-(4-hydroxyphenyl)-2-hydroxypropionic acid tri(TMS) derivative match the spectrum of the compound in urine. However, the spectrum is an exact match for the mass spectrum of a 3-(3-hydroxyphenyl)-3-hydroxypropionic acid tri (TMS) derivative (Fig. 1D) obtained from the laboratory that synthesized the compound. This compound was designated β-*p*-hydroxyphenylhydracrylic acid in older nomenclature. Furthermore, the compound does not match the spectra of authentic 3-(4-hydroxyphenyl)-3-hydroxypropionic acid tri (TMS) or 3-(3-hydroxyphenyl)-2-hydroxypropionic acid tri(TMS) derivatives obtained in the same laboratory.[8]

To identify the compound of interest in urine further, an identical urine extract was prepared and

Table 1Interpretation of mass spectra of urine compound derivative prepared with deuterated and non-deuterated BSTFA

Loss from 398	Major ions with TMS	Major ions with d_2 TMS	Δ	TMS content	Interpretation
325	73	82	9	1 TMS	TMS
251	147	162	15	1 TMS 1 DMS	TMS-O-DMS
131	267	285	18	2 TMS	M-CH_2COOTMS
118	280	298	18	2 TMS	M-COOHTMS
57	341	365	24	2 TMS 1 DMS	M-CO-CH_2-CH_3
15	383	407	24	2 TMS 1 DMS	M-CH_3
0	398	425	27	3 TMS	M

The values in columns 1–4 are in daltons.
Δ is the difference in molecular weight (Da) of major ions of the compound derivatized with deuterated BSTFA (column 3) compared to non-deuterated BSTFA (column 2). Thus, Δ = value in column 3 – value in column 2.
TMS, trimethylsilyl; DMS, dimethylsilyl.
In the last column, M is the molecular ion.

then derivatized with perdeuterated BSTFA. The ions in the mass spectrum of the non-deuterated derivative were then compared to the ions in the mass spectrum of the perdeuterated derivative (Table 1). Since the deuterium of perdeuterated BSA is covalently bonded to the TMS groups, the transfer of the perdeuterated TMS groups to the accepting molecule increases the mass of the resulting deuterated derivative in proportion to the number of functional groups derivatized. Functional groups derivatized by the derivatizing reagent include carboxyl, hydroxyl, sulfhydryl and amino groups. Thus the transfer of one deuterated TMS group would add 9 deuterium atoms to the derivatized molecule, increasing the mass by 9 Da compared to the derivative containing ordinary hydrogen. Two deuterated TMS units would add 18 Da, and so forth. The ion at *m*/*z* 398 in the spectrum of the non-deuterated TMS derivative shifted to *m*/*z* 425 (a shift of 27 Da) in the spectrum of the perdeuterated derivative indicating that this ion contained three TMS groups (27/9 = 3) and was likely the molecular ion. The ion at *m*/*z* 383 in the spectrum of the non-deuterated TMS derivative shifted to *m*/*z* 407 (a shift of *m*/*z* 24) in the spectrum of the perdeuterated TMS derivative indicating the presence of two TMS groups (shift of 18 Da) and one dimethylsilyl group (shift of 6 Da), indicating a loss of a methyl group from a TMS group, a loss extremely common in TMS derivatives. The ion at *m*/*z* 341 in the spectrum of the non-deuterated BSTFA derivative shifted to *m*/*z* 365 in the spectrum of the perdeuterated derivative consistent with migration of –OTMS combined with a loss of –CH_3 from a TMS group and a loss of CH_2CO. All of the data support the identification of the urine compound as 3-(3-hydroxyphenyl)-3-hydroxy-propionic acid tri (TMS) derivative.

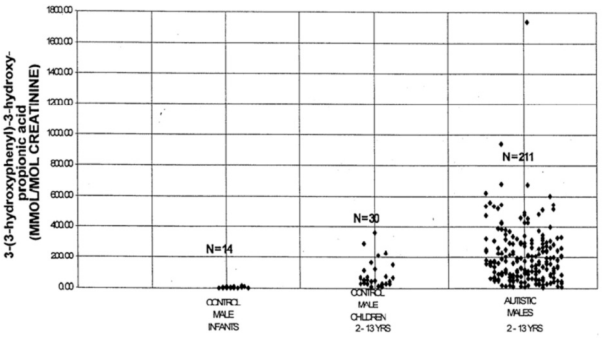

Figure 2 Distribution of 3-(3-hydroxyphenyl)-3-hydroxy-propionic values in urine samples of male infants, male control children, and male autistic children

With the high-resolution mass spectrometer, the mass of the putative molecular ion was measured three times, yielding an average of 398.1766 Da. The theoretical accurate mass for 3-(3-hydroxyphenyl)-3-hydroxy-propionic acid-TMS [3] is 398.1765 Da. The average of the measured values agree to within < 1 ppm of theoretical for

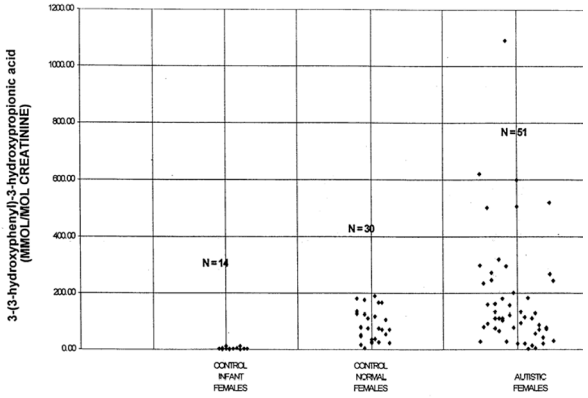

Figure 3 Distribution of 3-(3-hydroxyphenyl)-3-hydroxypropionic values in urine samples of female infants, female control children, and female autistic children

Table 2 Urinary excretion of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid in a drug-free patient with first onset of schizophrenia symptoms

Compound in urine of patient during acute psychosis (mmol/mol creatinine)	7500
Compound in urine of same patient 6 months after antimicrobial treatment (mmol/mol creatinine)	673
Compound in urine samples of normal adult controls (n = 19) (mmol/mol creatinine)	Mean 39.8, median 24.6; SD 50.2

an elemental composition of $C_{18}O_4Si_3H_{34}$, the elemental composition of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid or 3-(hydroxyphenyl)-3-hydroxypropionic acid TMS [3] derivative. The identity of the compound was later confirmed by comparison of both retention time and mass spectrum of the authentic compound.

Concentration of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid in urine samples of humans

The excretion of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid in urine was very low in a group of infants attending a well-baby clinic at 6 weeks of age (Figs 2 and 3). The mean value for all infants is 3.7 mmol/mol creatinine with a standard deviation of 3.6 mmol/mol creatinine and a range from 0.3–12.7 mmol/mol creatinine. In normal male control children, the mean value is 91.5 mmol/mol creatinine with a standard deviation of 90.4; the median value in this group is 51.1 mmol/mol creatinine. In autistic male children, the mean value is double that of the controls: 192.4 mmol/mol creatinine with a standard deviation of 90.4; the median value in the autistic male children group is 143.5 mmol/mol creatinine, nearly triple the value of the control group. In normal female control children, the mean value is 85.5 mmol/mol creatinine with a standard deviation of 55.9; the median value in this group is 74.5 mmol/mol creatinine. In autistic female children, the mean value is double that of the controls: 182.4 mmol/mol creatinine with a standard deviation of 200.6; the median value in this group is 111 mmol/mol creatinine, a value 49% greater than the control females. The differences between autism and control groups of the appropriate sex are statistically significant by the t-test at $P < 0.005$.

The highest value for this compound (Table 2) in over 7000 urine samples tested was a value of 7500 mmol/mol creatinine in a 21-year-old female with acute schizophrenia during an acute psychotic episode. Normal adult values (n = 19) for the compound are: mean, 39.8 mmol/mol creatinine; median, 24.6 mmol/mol creatinine; SD, 50.2 mmol/mol creatinine. Thus, the value in the urine of the schizophrenic patient was 300 times the median normal value. The patient was treated with oral vancomycin for one week, resulting in normalization of the psychotic behavior without the use of neuroleptic drugs. Re-testing the patient 6 months later indicated the compound in urine to be 673 mmol/mol creatinine, a value still significantly higher (nearly 27 times the median normal value) than normal values. Presumably, some recolonization of the *Clostridia* may have occurred after antimicrobial treatment ceased. The value in a child with child psychosis during hospitalization was probably even greater than that in this schizophrenic patient since the peak in the patient with child psychosis was so large it obscured about half the chromatogram. However, exact quantitation was not done on this patient.

Effect of metronidazole on urinary excretion of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid

Testing was performed on several patients at the attending physician's request who had suspected or confirmed clostridial infections and were treated with metronidazole, an antibacterial agent with specificity toward anaerobic bacteria and no antifungal properties.[9,10] I tested several of these patients before and after metronidazole therapy at standard age-appropriate dosages and found a substantial decrease in the concentration of this compound from baseline in these patients after drug therapy (Table 2). As shown in Table 3, there is a marked decrease in the urinary concentration of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid following the oral administration of the antibiotic metronidazole at 50 mg/kg/24-h divided into three doses. In all four patients, the concentrations of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid decreased 99% or more after 2–3 weeks on this drug. In the first patient in the above series, 3-(3-hydroxyphenyl)-3-hydroxypropionic acid rapidly increased following the cessation of metronidazole treatment. There was a severe Herxheimer or 'die-off' reaction (toxin release as the bacteria die) for several days with the use of this drug that includes fever, lethargy, profuse sweating, and heart palpitations.

Discussion

The marked decrease in 3-(3-hydroxyphenyl)-3-hydroxypropionic acid (Table 2) following treatment with metronidazole is consistent with the production of this

Table 3 Effect of metronidazole therapy on urinary excretion of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid

Diagnosis and sex	Age (years)	Duration of time metronidazole therapy (days)	Urinary 3-(3 hydroxyphenyl)-3-hydroxypropionic acid (mmol/mol creatinine)
Autism, male	4	0	435
		6	184
		16	1
		21 (stop metronidazole)	5
		24	2
		43	236
		93	274
Previous *C. difficile* infection and uncontrolled diarrhea, adult female	54	0	396
		13	1
Autism, male	3	0	549
		19	1
		30	3
Autism, male	4	0	1362
		11	28
		15	3

compound by one or more species of anaerobic bacteria. Phenylpropionic acid and/or monohydroxyphenylpropionic acid, which are very closely related biochemically to this compound, are produced by multiple species of *Clostridia* including *C. sporogenes*, *C. botulinum*, *C. caloritolerans*, *C. mangenoti*, *C. ghoni*, *C. bifermentans*, *C. difficile*, and *C. sordellii* while *C. tetani*, *C. sticklandii*, *C. lituseburense*, *C. subterminale*, *C. putifaciens*, *C. propionicum*, *C. malenomenatum*, *C. limosum*, *C. lentoputrescens*, *C. tetanomorphum*, *C. coclearium*, *C. histolyticum*, *C. aminovalericum*, and *C. sporospheroides* do not produce these compounds.[11]. Bhala *et al*.[12] found that *Clostridia* were the only organisms that produced phenylpropionic acid after they evaluated 67 different isolates of microbes from nine different genera of bacteria and *Candida albicans*. Furthermore, they found that metronidazole, clindamycin, and combined therapy of ticarcillin, clavulanate, and oxacillin abolished gut flora producing phenylpropionic acid. Cefalzolin, cefuroxime, ampicillin, chloramphenicol, and gentamicin did not abolish phenylpropionic production. Since a large group of intestinal bacteria including *E. coli*, *Streptococci*, *Salmonella*, *Shigella*, *Proteus*, *Pseudomonas*, and *Klebsiella* are killed by one or more of these agents,[13] the persistence of phenylpropionic acid in the presence of these agents appears to eliminate them as potential sources of this compound. (This latter group of drugs is generally ineffective against *Clostridia* species in mixed cultures like those in the gastrointestinal tract; the other species may inactivate antibiotics such as penicillin even though *Clostridia* in pure cultures may be susceptible to these antibiotics.) The increase in 3-(3-hydroxyphenyl)-3-hydroxypropionic acid in the urine of the child with autism after cessation of metronidazole treatment (Table 2) is consistent with the frequent recurrence of gastrointestinal *Clostridia* due to germination of resistant spores following antibiotic treatment. Alternatively, drug-resistant organisms may have been present that might have required longer therapy or the dose of drug may not have been adequate for total clearance of the organism.

I did not find 3-(3-hydroxyphenyl)-3-hydroxypropionic acid in multiple culture media samples in which multiple species of *Clostridia* were cultured. The lack of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid in pure cultures of *Clostridia* is almost surely due to the production of precursors of the compound such as phenylpropionic and monohydroxyphenylpropionic acids that are then converted to 3-(3-hydroxyphenyl)-3-hydroxypropionic acid (Fig. 4) by human metabolism.

The origin of this compound (Fig. 4) is almost surely dietary phenylalanine in the intestinal tract that is converted to 3-hydroxyphenylpropionic acid by two possible routes. The hydroxylation of the phenylalanine ring at the 3-position may occur before or after removal of the amino group (deamination). If deamination occurs first, phenylpropionic acid would be formed. If deamination occurs after hydroxylation, *m*-tyrosine (3-hydroxyphenylalanine) would be formed (Fig. 4). *m*-Tyrosine induces a characteristic behavioral syndrome in rats consisting of forepaw padding, head weaving, backward walking, splayed hind limbs, wet dog shakes, hyperactivity and hyper-reactivity and depletes the brain of catecholamines.[14] Thus, this compound might play a direct role in causing abnormal behaviors in autism, schizophrenia, and other disorders. It is also possible that this compound might form an analog of dopamine, if *m*-

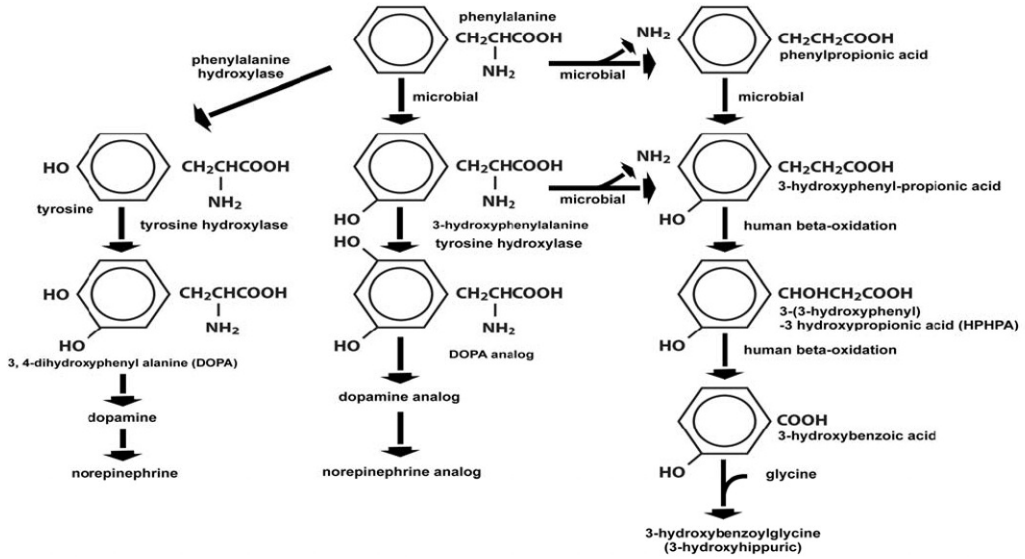

Figure 4 Suggested pathway for the metabolism of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid

tyrosine is metabolized by the same enzymes that convert tyrosine to dopamine.

Male and female autistic children both excreted more 3-(3-hydroxyphenyl)-3-hydroxypropionic acid than the appropriate control group. The difference is especially prominent in autistic males in which the median value is nearly triple that of the control children. The other metabolic routes for this compound might be expected to include sulfation and/or glucuronidation of the phenolic groups (Fig. 4). A similar compound, 3-phenylpropionic acid, is converted to benzoic acid by the enzymes of fatty acid oxidation.[15] These enzymes convert phenylpropionic acid to benzoic acid, which is then conjugated with glycine in the liver to form hippuric acid (Fig. 4). If these same enzymes metabolize 3-(3-hydroxyphenyl)-3-hydroxypropionic acid, increased hydroxybenzoic acid and its glycine conjugate hydroxyhippuric acid would be expected to be formed when excessive 3-(3-hydroxyphenyl)-3-hydroxypropionic acid is produced. Hydroxyhippuric acid TMS derivative, usually a very small peak in urine extracts of normal individuals, is indeed present as a large peak in the chromatogram of the urine sample extract of the child with autism in Figure 1A. The finding of elevated median values of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid in urine samples of autistic children might indicate they harbor bacteria that produce more of this compound than in normal children. Previous studies have indicated lower activity of phenolsulfotransferase in autistic children,[16] which might result in decreased detoxification of this compound by the sulfation pathway characteristic of phenolic compounds.

This compound might also have importance as a marker for the overgrowth of *Clostridia* in the gastrointestinal tract. The very low values of this compound in both male and female infants (Figs 2 and 3) are consistent with the age at which *Clostridia* normally colonize the gastrointestinal tract.[16] Breast-fed infants are colonized with almost no *Clostridia* but extensive anaerobic bacterial colonization begins with the introduction of solid foods.[17] Bennett *et al*.[18] found that phenylpropionic acid was not detected in 84% of stool samples from infants younger than 4 months but was present in 67% of stool samples from infants 4 months or older. Intestinal overgrowth of *C. botulinum* and *C. difficile* both cause serious illness,[19] and *C. botulinum* in infant botulism produces a neurotoxin that is apparently absorbed from the gastrointestinal tract.[20] Increased frequency of ear infections in children with autism has been documented and the severity of the autism has been related to both the frequency of such ear infections and the age of onset of ear infections.[2] Since many species of *Clostridia* are not susceptible to some of the common antibiotics used to treat ear infections, antibiotic therapy might be selecting harmful species of *Clostridia*. It is also possible that these children may be susceptible to harmful *Clostridia* as a consequence of their environmental exposure in early life and/or their specific genetic make-up, enabling these organisms to proliferate.

Phenylpropionic acid has only been detected in the culture media of bacterial species of the *Clostridium* genus,[18] indicating that a closely related compound, 3-

(3-hydroxyphenyl)-3-hydroxypropionic acid is probably a product of *Clostridia* species. I am currently examining stool samples of autistic children to determine if there are certain species of *Clostridia* that are prevalent in these children. Bolte[20] has hypothesized that gastrointestinal tetanus due to *C. tetani* may be a major etiological agent in autism and compares symptoms of autism to subacute gastrointestinal tetanus infection; animals with tetanus exhibit many of the same characteristics as individuals with autism: stereotyped behavior, hypotonia, difficulties in chewing and swallowing, reduced learning ability, and seizures. Sandler *et al*.[21] reported that vancomycin treatment of a group of autistic children resulted in a significant decrease in autistic symptoms. However, the benefits of therapy were lost after vancomycin treatment ended. This regression is consistent with possible *Clostridia* overgrowth of the intestinal tract in which *Clostridia* commonly recur after discontinuation of antibiotic use because of formation of resistant spores.[22] Bolte[20] demonstrated that a similar number of *Clostridia* species was harbored by autistic spectrum disorder (ASD) patients and healthy controls. However, nine *Clostridium* species were exclusively isolated from stool samples of autistic children (*i.e*. not found in the predominant fecal microflora of healthy controls). In addition, three species were only found in healthy samples. In a subsequent study, Song *et al*.[23] identified significantly higher levels of *C. bolteae* and *Clostridium* clusters I and XI in autistic children than in healthy controls. The fecal flora of patients with ASDs was studied by Helena *et al*.[24] and compared with those of two control groups (healthy siblings and unrelated healthy children). Fecal bacterial populations were assessed through the use of a culture-independent technique, fluorescence *in situ* hybridization, using oligonucleotide probes targeting predominant components of the gut flora. The fecal flora of ASD patients contained a higher prevalence of the *C. histolyticum* group (*Clostridium* clusters I and II) of bacteria than that of healthy children.

Phenylpropionic acid, the probable precursor of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid, is an *in vitro* inhibitor of both carboxypeptidase and enkephalinase activities;[25] administration of this compound to mice raises brain enkephalin concentrations[26] and causes analgesia when injected intraperitoneally into mice. I have detected phenylpropionic acid in many of the same urine samples in which 3-(3-hydroxyphenyl)-3-hydroxypropionic acid is elevated, and I suspect that elevation of enkephalins due to enkephalinase inhibition by phenylpropionic acid in humans might also contribute to abnormal behaviors. An evaluation of the possible role of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid in inhibiting these same enzymes may be worthwhile because of the biochemical similarity of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid and phenylpropionic acid.

High doses of the GG strain of *Lactobacillus acidophilus* have been used to control *C. difficile*.[27] *L. acidophilus* therapy has no reported toxicity, and treatment with *L. acidophilus* GG of individuals with an elevated concentration of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid in their urine markedly reduces the concentration of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid in subsequent urine samples (unpublished data). Bolte[20] reported a marked decrease in symptoms of autism in children treated with antibiotics effective against *Clostridia*, indicating treatment of abnormal microbial overgrowth may be a promising new therapy for the treatment of autism in individuals with this abnormality. The observation that elevated amounts of this compound in urine samples were associated with mental illnesses in general was made 50 years ago but has been completely ignored since then.[28] Significant decreases in symptoms of schizophrenia, tic disorders, depression, chronic fatigue syndrome, and attention deficit hyperactivity have been reported by the attending physicians (personal communications, see Addendum) following antimicrobial treatment of individuals with elevated urinary concentrations of this compound, indicating that this compound may be of importance to many other mental diseases in addition to autism but also indicating that these probable *Clostridia* species are not specific for the etiology of autism or other diseases.

3,4-Dihydroxyphenylpropionic acid (DHPPA), a compound I found at very low amounts in the urine, is a by-product of chlorogenic acid, a common substance found in beverages and in many fruits and vegetables including apples, pears, tea, coffee, sunflower seeds, carrots, blueberries, cherries, potatoes, tomatoes, eggplant, sweet potatoes, and peaches.[29–40] Because of the chemical similarities, similar retention times in many chromatographic systems, and the similar mass spectra of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid (HPHPA) and DHPPA, it is important to differentiate the sources of these compounds. The breakdown of chlorogenic acid is mediated mainly by harmless or beneficial bacteria such as *Lactobacilli*, *Bifidobacteria*, and *E. coli*.[41]. In addition, one clostridial species, *C. orbiscindens*, can convert the flavanoids luteolin and

eriodictyol, that occur only in a relatively small food group that includes parsley, thyme, celery, and sweet red pepper to 3,4-dihydroxyphenylpropionic acid.[42] In addition, DHPPA is also produced from wine phenols and catechin, a constituent in chocolate. The quantity of *C. orbiscindens* in the gastrointestinal tract is negligible (approximately 0.1% of the total bacteria) compared to the predominant flora of *Lactobacilli*, *Bifidobacteria*, and *E. coli*.[43] Thus, elevated amounts of this compound are due primarily to beneficial microbial breakdown of chlorogenic acid which is present in abundance in common major foods and is overwhelmingly an indicator of beneficial bacteria presence and/or a diet high in foods containing phenolic flavanoid compounds.

Elevated values of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid (data not shown) also occur in children with attention deficit hyperactivity and child psychosis, adults with depression, and in some children and adults with seizure disorders, chronic fatigue syndrome, obsessive-compulsive disorders, and tic disorders (unpublished data). The compound is not drug derived since it is found in the urine of many drug-free persons, including the drug-free schizophrenic patients obtained from Dr Gattaz.

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Addendum

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