

Increased Urinary Excretion of Analogs of Krebs Cycle Metabolites and Arabinose in Two Brothers with Autistic Features

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Abstract

A marked increase in abnormal Krebs cycle metabolites was found in the urine of two brothers with autistic features. These metabolites include citramalic, tartaric (3-OH-malic), and 3-oxoglutaric acids, and compounds tentatively identified as a citric acid analog and partially identified as a phenylcarboxylic acid by the fragmentation pattern of the trimethylsilyl (TMS) derivatives of the compounds and mass shifts of the same compounds derivatized with perdeuterated N,O-bis (trimethylsilyl) trifluoroacetamide. The molecular weight of the TMS derivative of the tentatively identified citric acid analog is 596 Da based on finding a significant M-15 ion at m/z 581. The concentration of the citric acid analog was excreted in quantities as high as 137 mmol/mol creatinine based on the response factor of citric acid as a surrogate calibration standard. A carbohydrate with a retention time and mass spectrum identical to arabinose was also found in high concentrations in the urine of these brothers.

Abbreviations:

PDD - pervasive developmental disorder

DMS - dimethylsilyl

GC/MS - gas chromatography-mass spectrometry

CoA - coenzyme A

TMS - trimethylsilyl

EI - electron impact

BSTFA N,O - bis(trimethylsilyl)acetamide

CSF - cerebrospinal fluid

GRAS - generally recognized as safe

INTRODUCTION

Childhood autism is the most characteristic group of the broader pervasive developmental disorder (PDD) category. The cause of this disorder is unknown except for a small subgroup due to adenylosuccinic aciduria, a defect in purine metabolism (1-3). Autism is characterized by a behavioral syndrome that is recognized between 2 and 3 years of age. The core of the syndrome is a deviant and/or retarded development of cognitive capacities and skills necessary for social relations, communication, fantasy, and symbolic thinking. Almost all autistic people would not reach independence as adults and 75% are mentally retarded as well (4). Taurine, aspartate, and glutamate are reported to be significantly elevated in the plasma of a significant fraction of autistic persons and some have metabolic acidosis (5).

Our interest in a possible metabolic cause for autism was stimulated by referral of two brothers with autistic features. The older brother was referred at 10 years for evaluation of autistic features. He had normal development until 20 months when he then appeared to remain at a standstill followed by loss of

previously acquired skills. He had frequent ear infections during infancy and was treated with antibiotics.

Eventually he demonstrated decreased cognitive function, short attention span, aggressive behavior, difficulty making transitions, and a fascination working with arithmetic problems. At 30 months speech and language functions deteriorated progressively and by 36 months he was noted to lose muscle strength intermittently, lasting for several hours during which he was weak enough to fall. These episodes did not always coincide with periods of ketosis. A muscle biopsy showed normal structural features except for unexplained "granularity" by electron microscopy. At age 4 years his behavior became frankly autistic and was so labeled in a major psychiatric center. Laboratory studies, including electroencephalograms, brain scans (both computerized tomography and magnetic resonance imaging), electromyography and nerve conduction velocities, and various blood and urine investigations, including lactate, pyruvate, ammonia, glucose, amino acids, carnitine, thyroid function, adenylosuccinic acid in urine and ceruloplasmin determinations were unremarkable except for the presence of increased 3-OH-butyric acid when urine was positive for ketones by dipstick (Keto-stix R).

Repeated urine organic acid tests in this laboratory eventually resulted in consistent findings of increased concentrations of metabolites that were not associated with any known metabolic disease. Because of these abnormalities an empirical trial of thiamine (25 mg daily) and pyridoxone (50 mg daily) was given, as well as some dietary modifications including avoiding red

meat, increasing the intake of complex carbohydrates, and eating more frequent small meals. On this regimen the episodes of ketosis and weakness decreased in frequency and severity. An 8-year-old brother appeared to show similar clinical features as the sibling, although not as severe. As an infant his mother described him as irritable, crying, and "not cuddly". He developed vomiting and required several formula changes. He also had frequent episodes of otitis media during infancy treated with antibiotic therapy.

His initial motor development was normal and he sat at 7 months, walked by 11 months, and said single words at 14 months. Cognitive, motor and language function began to regress and eventually he lost ability to speak. Similar investigations as done on his older brother were also unremarkable except for the presence of abnormal metabolites in the urine organic acid analysis that were not associated with any known metabolic disease.

MATERIALS AND METHODS

Gas chromatography-mass spectrometry(GC/MS). Urinary organic acids were quantified as their trimethylsilyl (TMS) ethers or esters essentially as described by Tanaka et al(6). Before acidification and solvent extraction, 1 mL of urine was incubated with 200 μ L of ethoxylamine hydrochloride solution at a concentration of 75 g/L at 60°C for 30 minutes to convert ketoacids to their ethoxime derivatives (7). In order to identify unknown compounds, the same procedure was performed except that perdeuterated N,O-bis(trimethylsilyl) acetamide (BSTFA) was substituted for ordinary BSTFA. In one experiment methoxylamine at the same concentration as ethoxylamine was substituted in the standard

procedure. All analytical standards were purchased from Sigma Chemical Co., St. Louis, MO. Quantitation for compounds for which no analytical standards were available were performed by assigning the response of an average size ion chromatogram peak as 100 UNITS and then calibrating all other peaks against these arbitrary standards. The GC/MS system used was from Hewlett Packard, Palo Alto, CA and consisted of an HP5970 mass selective detector, a 5890A gas chromatograph, a model 18593B autosampler, and an Apollo 400 series computer with a 664 MB hard disk drive. The operating software for both instrument control and data analysis was a UNIX-based Chemsystem which operated simultaneously with a Target 2 software system. For GC/MS analysis, 1 μ L of sample was injected onto a 15-meter, DB-1 capillary column with a 0.25 mm internal diameter and an 0.25 micron film from J & W Scientific, Folsom, CA, using purified helium as the carrier gas. All experiments were done using electron-impact (EI) ionization with electron energy of 70 eV. The temperature program was started at 90° C, held for 4 minutes after injection and then increased to 280°C at a rate of 8° C/min. The electron-impact mass spectra of arabinose and arabitol were differentiated by the fact that the spectrum of the TMS derivative of arabitol has a significant ion at m/z 319 that is not present in the spectrum of the TMS derivative of arabinose.

Urine samples were randomly collected in plastic screw-cap containers and stored at -20° C until tested. Normal urine samples were collected from children of laboratory employees. Urine creatinine tests were performed by a modification of the Jaffe method (8) on an automated chemistry analyzer. The

procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 1983.

RESULTS

A typical total ion current (TIC) chromatogram of the TMS derivatives in the urine of brother A is shown in (Figure 1A). Significant peaks at 10.31 min, 14.36 min, 17.78 min, and 18.86 minutes were present that were not prominent in normal urine samples (Figure 1B). The results of the urine samples of brother B were very similar to those of his sibling A. In addition, tartaric acid was frequently found in high concentrations in urine samples of these siblings. A much smaller peak at 12.1 min was also detected in some of the urine samples of the siblings with autistic features. Since the concentration of these metabolites in urine samples of the siblings vary widely, we think that it is very important to show data demonstrating the variability of the concentration of these compounds and comparing these concentrations to those in normal children. Therefore, results for four of the metabolites are presented in an **identification section** and a **quantitation section** comparing them to normals. No identification section is given for tartaric acid since it is well-recognized in the field of metabolic diseases. No quantitation section is given for 3-oxoglutaric acid since it was only quantitated in some of the samples.

Tentative identification of 2-methylmalic(citramalic) and 3-methylmalic acids

The mass spectrum of the peak at 10.31 min (Figure 2A) is very similar to that of authentic citramalic acid (2-hydroxy-2-methylbutanedioic or 2-methylmalic acid (Figure 2B) and has an identical retention time (± 0.1 min). However, a very abundant ion at m/z 205 and a less abundant ion at m/z 220 were present in the spectrum of the peak in the urine of the child with autistic features. The abundance of the ions at m/z 205 and m/z 220 varied widely in different urine samples of the brothers with autistic features. For example, in a spectrum from a different urine extract the abundances of the ions at m/z 203 and 205 are approximately equal and the 220 ion is not detectable (Figure 2C). We suspect that the compound producing the 205 and 220 ions is 2-hydroxy-3-methyl-butanedioic (3-methylmalic). The abundant ion fragment at m/z 220 would be consistent with the loss of a $\text{CH}_3\text{-C-CO}_2\text{TMS}$ fragment following a McLafferty rearrangement and cleavage of the bond between carbons 2 and 3; a loss of CH_3 from another TMS group from the m/z 220 ion fragment yields the ion fragment at m/z 205 (Figure 3). Equivalent ion fragments cannot be formed by fragmentation of 2-methylmalic. 3-Methylmalic acid is not commercially available for confirmation studies.

Quantitation of methylmalic acids

For our quantitative studies, the ion signal peak at m/z 247 was used for quantitation. Since both 2- and 3-methylmalic acid isomers have abundant 247 ions and we used citramalic acid(2-methylmalic) as the calibration standard, the

values we give for citramalic acid probably represent the total of these isomers. Concentrations of this compound are markedly higher in the brothers with autistic features than in normal children. Citramalic acid concentrations in 19 of the 20 urines from normal children are below 2 mmol/mol creatinine while citramalic acid concentrations in 14 of the 15 urines from the brothers with autistic features exceed 2 mmol/mol creatinine and in one of the urine samples from brother A is 62 mmol/mol creatinine (Figure 4). The mean citramalic acid concentration in the brothers with autistic features is 14.4 mmol/mol creatinine and 1.5 mmol/mol creatinine in the normal children. The means are statistically different using the t-test at the 0.01 probability level.

Tentative Identification of new citric acid analog.

The possibility that a new citric acid analog might be present in the urine of autistic children was entertained when an analysis of the mass spectrum of the peak eluting at 17.78 minutes (Figure 5 C) following the computerized mass spectra library search revealed that the unknown shared several unique ions with the TMS derivatives of citric and isocitric acids (Figure 5 A, B) including those for m/z 273, 347, and 375. Selected ion chromatograms of multiple urine extracts revealed that all of these three ions were found only in peaks corresponding to the TMS derivatives of citric, isocitric, and the unknown peak. The unknown spectra had a significant ion at m/z 581. Since the largest detectable ion for TMS derivatives is frequently the M-15 ion due to the loss of a methyl group from one of the TMS groups, the tentative molecular weight of

596 could correspond to a citric acid TMS derivative (molecular weight = 480 Da) with an additional COOTMS group (mass = 117 Da) - H (mass = 1 Da).

Additional evidence for a citric acid analog was obtained by making a perdeuterated TMS derivative of the compound. An aliquot of the same urine was extracted by the regular method and then derivatized with perdeuterated BSTFA to identify the fragments, mass losses from the molecular ion, TMS and/or dimethylsilyl (DMS) content of each mass fragment and the number of functional groups. The mass spectra of the compound formed with the perdeuterated BSTFA is given in Figure 5D and an interpretation of the data from the spectra of both the perdeuterated and nondeuterated BSTFA derivatives are given in Table 1.

The largest mass fragment in the spectrum of the nondeuterated compound is m/z 581 Da ; the largest mass fragment in the perdeuterated derivative is m/z 623, a shift of 42 Da. This mass shift is consistent with the 581 Da fragment ion containing five derivatized functional groups, four of which are TMS and one of which is DMS. These data also indicate that the m/z 581 ion is the M-15 ion, and that the molecular weight of the penta TMS derivative is 596 Da. The ion at m/z 491 in the spectrum of the nondeuterated compound corresponds to the ion at m/z 524 in the spectrum of the perdeuterated derivative, a shift of 33 Da, indicating that this ion contains three TMS and one DMS groups, and that one TMS group and a methyl group from one TMS group were lost from the molecular ion in the formation of this ion. This ion at m/z 491 results from a loss of 105 Da from the molecular ion; one TMS accounts for 73

Da and the methyl group accounts for 15 Da, leaving a mass of 17 Da unaccounted. This remaining mass of 17 Da is consistent with the loss of OH, as TMSOH, an extremely common loss in the literature of the mass spectra of TMS derivatives(9)..

The ion at m/z 463 in the spectrum of the nondeuterated compound corresponds to the ion at m/z 496 in the spectrum of the perdeuterated derivative, a shift of 33 Da, indicating that this ion contains three TMS and one DMS groups and that one TMS group and a methyl group from another TMS group were lost in the formation of this ion. The ion at m/z 463 results from a loss of 133 Da from the molecular ion; one TMS accounts for 73 Da and the methyl group from another TMS accounts for 15 Da, leaving a mass of 45 Da unaccounted, which clearly is consistent with the loss of COOH, as TMSCOOH, an extremely common loss in the literature of the mass spectra of TMS derivatives (9).

The ion at m/z 421 in the spectrum of the nondeuterated compound corresponds to the ion at m/z 457 in the spectrum of the perdeuterated derivative, a shift of 36 Da, indicating that this ion contains four TMS groups and that one TMS group was lost in the formation of this ion. The ion at m/z 421 is unusual in that one COO is lost without an accompanying TMS group, a neutral loss of 175 from the molecular ion (Figure 6 and Table 1). This ion (Figure 6) is attributed to the loss of CH_2COOTMS (-131) followed by a rearrangement of the COOTMS group attached to carbon-3 in which the silicon atom of this COOTMS group attacks carbon-3 and CO_2 is expelled(-44). Similar losses of 175, also

found in the mass spectra of citric and isocitric acids, are consistent with the proposition that the structures (Figure 6) for the three compounds are similar and result in similar fragmentation patterns. This loss of 175 can occur in two different ways in citric acid since structure a and structure b (Figure 6) are equivalent. In isocitric acid, this loss of 175 can only involve structure b of the molecule. The molecular weight of the unknown compound is 596 Da. The fragmentation pattern and labeling prove the presence of at least one OTMS group and two COOTMS groups, accounting for 323 Da. Two additional TMS groups account for 146 Da. The results of the labeling experiments with the unknown and the similar fragmentation patterns of the unknown, citric acid, and isocitric acid are consistent with identical portions of these molecules labeled structure b in Figure 6. The carbon atom at C-3 and the CH₂ group in structure b in Figure 6 account for an additional 26 Da, leaving 101 Da unaccounted; the two additional functional groups remaining can only be COOTMS and TMSOH since there are no losses consistent with any other functional groups. Thus, the rest of the molecule contains two COOTMS groups, or two OTMS groups, or one OTMS group and one COOTMS group. The presence of two additional OTMS groups is unlikely because there are no ions that correspond to the loss of two OTMS groups. Thus, the unknown compound is likely a citric acid analog with an extra carboxylic acid group or a hydroxycitric acid. Because the unknown is more unstable than citric acid, we suggest that a 2-carboxycitric acid structure is more likely. 2-carboxycarboxylic acids are relatively unstable (Dr. Marty Powers, Midwest Research Institute, Kansas City, MO).

Because this derivative was formed in the presence of ethoxylamine HCl, we wished to rule out the possibility that the compound is an ethoxime derivative. Substitution of methoxylamine HCl in the procedure yielded a derivative with an identical spectrum indicating that no oxime was present in the molecule, and therefore no keto group was present in this compound. Curiously, when an identical urine aliquot was tested with the oxime derivatization step omitted, this compound was not detected. We suspected that the failure to detect this compound was due to the salt effect of the methoxylamine HCl or ethoxylamine HCl which increased the efficiency of extracting an extremely water-soluble compound with five polar functional groups into the organic solvents.

We attempted to confirm this idea by substituting sodium chloride for the oxime reagent and then performing the normal extraction and derivatization procedure. Results of this experiment revealed that this compound was still not detected, indicating that the effect of the oxime is not a simple salting out effect but might be the result of ion pair formation between the positively charged oxime ions and the negatively charged citric acid analog. We also note that the size of the peak for citric acid was also markedly diminished when oxime reagents were omitted also strengthening the hypothesis that the oxime reagent acts as an ion pair extraction reagent for other highly water-soluble acids. However, 2- carboxycarboxylic acids are very unstable, especially in acidic solution and tend to decarboxylate (Marty Powers Ph.D., Midwest Research Institute, Kansas City, MO) and the oxime might also protect this molecule from

decarboxylation. We are currently setting up other studies in which other amines are substituted for ethoxylamine to determine if other positively charged counter ions possessing a hydrophobic moiety could form ion pairs that would be effectively extracted.

Complete confirmation of the structure of this compound requires purification and isolation of the molecule followed by high resolution mass-spectrometry, NMR spectroscopy, elemental analysis, and chemical synthesis. This work is now in progress.

Quantitation of tentatively identified citric acid analog

The tentatively identified citric acid analog is found in much higher values in the urine of the brothers with autistic features compared to normal children (Figure 7). The mean value for the brothers with autistic features is 93 units/mol creatinine while the mean value for normal children is 18 units/mol creatinine. Brother A excreted the largest amount of (335 units/mol creatinine). If this compound has a response factor for total ion current equivalent to citric acid, the absolute concentration would be 137 mmol/mol creatinine. The presence of this compound in the urine of 18/20 normal children was confirmed by identification with complete mass spectra demonstrating that this compound is not a drug metabolite.

Identification of 3-oxoglutaric acid

3-Oxoglutaric acid in the urine of these brothers was identified by the fact that the retention time of the peak at 12.1 min by GC/MS and the spectrum of

the ethoxylamine-TMS derivative were similar to that of authentic 3-oxoglutaric acid processed by the standard method. In addition, the ion ratio for masses 318/243 is characteristic of 3-oxoglutaric acid. The ion ratio for 2-oxoglutaric acid is markedly different. However, the concentration of this compound was too low to obtain a conclusive mass spectrum. The urine samples of the brothers were also unusual in that the concentration of 3-oxoglutaric acid was nearly as great as that of 2-oxoglutaric acid, a finding confirmed in other autistic children. (A full description of the findings in other autistic children is in preparation.) The urine of another unrelated autistic child had a higher concentration of this compound which permitted unequivocal identification of this compound (Figure 8A). 3-Oxoglutaric acid elutes 0.3 min before 2-oxoglutaric acid with our chromatographic conditions. The mass spectrum of this compound is consistent with the presence of a di-TMS ethoxylamine derivative. Mass spectra for the two compounds are very similar. Both contain significant ions at m/z 318 due to loss of a methyl group from the molecular ion and significant ions at m/z 103 and 318. The spectrum of the 2-oxoglutaric acid derivative has prominent ions at m/z 288 and 198 (Figure 8C) that are not significant in the spectrum of the 3-oxoglutaric acid derivative (Figure 8B). The fragment at m/z 243 is abundant in the spectrum of 3-oxoglutaric acid but very weak in the spectrum of 2-oxoglutaric. A weak molecular ion at m/z 333 was identified in the spectra of both compounds. 3-Oxoglutaric acid was variably present in the urine of these brothers and was present as a relatively small peak that might not be

detected without the use of reconstructed ion chromatograms for m/z 243. The concentration in the child with the highest value was 26 mmol/mol creatinine.

Quantitation of tartaric acid. The distributions of tartaric acid concentrations are clearly different for the normal children and the brothers with autistic features (Figure 9). Tartaric acid exceeds 20 mmol/mol creatinine in only 6 of 20 (30%) of the normal urines while tartaric acid is above this value in 15/17 (88%) of the urines from the brothers with autistic features. The mean value in the normal children is 26.6 mmol/mol creatinine (SD = 26.6) while the mean value for the brothers with autistic features was 69.2 mmol/mol creatinine (SD = 71.3). However, an inspection of the data clearly indicates a non-normal distribution of data for which median values provide a more meaningful comparison of the two groups. The median value of the normal urine group is 3 mmol/mol creatinine but is 36 mmol/mol creatinine in the brothers with autistic features.

Identification of a phenylcarboxylic acid

The large peak at 18.86 min has been found in extremely high concentrations in urine samples of the brothers with autistic features and in urine samples of some other children with autism and as a much smaller peak in most normal children. The spectrum of this compound is shown in Figure 10. Ions at both m/z 73 and 147 indicate that this compound has at least two TMS groups. Other prominent ions are m/z 155, 273, 299, 350, 375, and 390. The use of perdeuterated TMS derivatives of this compound provides additional information about this compound (Table 2). The prominent ion at m/z 91 is consistent with the presence of the tropylium ion. An ion at m/z 65 is consistent

with the loss of CH_2CH_2 from the tropylium ion. The ion at m/z 299 is due to the loss of a tropylium ion from the molecular ion at m/z 390. The ion at m/z 273 is consistent with a loss of COOTMS from the molecular ion, which clearly contains 2 TMS groups and, therefore, two functional groups. The additional functional group appears to be a hydroxyl group based on the ions at m/z 100 and 113. Thus, this compound is partially identified as a phenylcarboxylic acid. However, the exact structure and elemental composition must still be elucidated.

Quantitation of phenylcarboxylic acid compound

The concentration of the phenylcarboxylic acid was found in much higher values in the urine of the autistic brothers than in normal urine (Figure 11). The concentration of this compound in 13 of 20 normal children is less than 10 units/mol creatinine while the concentration was as high as 800 units/mol creatinine in brother A. This compound was confirmed by identification of complete mass spectra in all of the normal children demonstrating that this compound is not a drug metabolite.

Identification of arabinose

The peak at 14.36 min was identified as the TMS derivative of the carbohydrate arabinose based on comparison of its mass spectrum and retention time to those of the TMS derivative of the authentic compound (Figure 12). Both D- and L-arabinose have identical retention times and mass spectra so that we do not know which isomer is present

Quantitation of arabinose

The mean concentration of arabinose in urine samples of normal children was 60.4 mmol/mol creatinine but an examination of the data in Figure 13 shows that the data are not normally distributed since data points are much more frequent at the lower concentrations. The median value in the urine samples of normal children is 31.0 mmol/mol creatinine. The median value for the urine samples of the brothers with autistic features is 179 mmol/mol creatinine, nearly six times greater than the median value for the urine samples of the normal children. The mean value for the urine samples from the brothers with autistic features is 305 mmol/mol creatinine, five times the mean for the urine samples from normal children. The highest concentration of urine arabinose, 1008 mmol/mol creatinine was obtained in a sample from brother B.

Concentration of metabolites in maternal urine

There were no other full -siblings of these brothers to evaluate. A half-sibling by their father and a different mother is alive but the father declined to have his urine or that of the half-sibling tested. The urine of the mother also had a somewhat unusual organic acid pattern in that the concentration of 3-oxoglutaric acid (6.1 mmol/mol creatinine) exceeded that of 2-oxoglutaric acid (5.3 mmol/mol creatinine). This same abnormal ratio was also found in several of the urine samples of the two brothers. The concentrations of arabinose (239 mmol/mol creatinine), the citric acid analog (74 mmol/mol creatinine), and the phenylcarboxylic acid compound(117 mmol/mol creatinine) were all elevated compared to normal children. The mother had no symptoms of autism.

Discussion

The structures of a number of compounds evaluated in this study are given in Figure 14. The citric acid analog, tartaric, citramalic, and 3-oxoglutaric acid are all analogs of Krebs cycle intermediates. (The structure of the citric acid analog is represented as 2-carboxycitric although the position of the extra carboxyl group has not been established.)

Elevated concentrations of citramalic acid appear to be clearly different than those in normal children since only one of the control children had a value greater than 2 mmol/mol creatinine. Citramalic acid, (2-methylmalic acid) and 3-methylmalic acid are analogs of the Krebs cycle intermediate malic acid and might interfere in the further metabolism of malic acid, leading to depletion of oxalacetic, the product of the action of malate dehydrogenase on malic acid. Oxalacetic acid is needed for condensation with acetic acid to replenish the Krebs cycle. A significant decrease in Krebs cycle activity could significantly impair cellular energy production. Very little information is available in the literature on citramalic acid or 3-methylmalic acid in biological fluids of humans. (A single reference to citramalic acid in a Medline search over the last 20 years was found.) Citramalic acid was found in increased concentration in cerebrospinal fluid (CSF) samples of patients with bacterial meningitis but not in normal samples of CSF, CSF samples from febrile patients or from patients with aseptic meningitis (10). Citramalic acid has not been reported as a mammalian metabolite but has been reported to accumulate in respiration-deficient mutants of brewer's, baker's, and wine yeasts (11). Furthermore, an

enzyme which catalyses the condensation of acetyl Coenzyme A (CoA) and pyruvate to form citramalic acid has been isolated from Baker's yeast (11). In addition, we have found citramalic to be consistently produced by *Propionobacteria acnes* cultures from human stool samples (unpublished data). A very large number of culture media from a wide variety of anaerobic bacteria isolated from stool samples by us were negative for citramalic acid production (unpublished data). It should also be noted that, although citramalic acid has not been reported as a mammalian metabolite, the possibility that it is of human origin cannot be ruled out.

The citric acid acid analog is a tentatively identified new molecule which was not previously known. The biosynthesis of several citric acid analogs has been reported as an ability of several species of fungi although the citric acid analog is not one of the analogs reported to be produced by these species (12). A citric acid analog methylcitric acid, is produced in the disease propionic acidemia when propionyl CoA instead of acetyl CoA condenses with oxalacetic acid (13). Carboxycitric acid could hypothetically be formed by condensation of malonyl CoA instead of acetyl CoA with oxalacetic acid, resulting in the production of 2-carboxycitric acid (Figure 15).

Tartaric acid is another compound that appears to be abnormally elevated. However, unlike citramalic, some normal children excreted significant amounts of tartaric acid. Tartaric acid is an analog of malic acid and is a known inhibitor of the citric acid cycle enzyme fumarase (14), which catalyses the interconversion of malate and fumarate. Tartaric acid is not known as a

mammalian metabolite. It is most widely known as a byproduct of the wine industry in which special procedures are used to remove tartaric acid sediment. Tartaric acid is known as a metabolic product of *Saccharomyces*. Since this species is endogenous to grapes, it is not clear whether all tartaric acid is a yeast metabolic product or whether some is due to endogenous grape metabolism (15). Tartaric acid is present in all grape products such as grape juice, wine, grape jelly, and is used as a food additive (16). However, grape products were not commonly ingested by either of the siblings with autistic features. It is classified as GRAS (generally recognized as safe) by the United States Food and Drug Administration (16). Evidence of toxicity is conflicting. It has been reported to cause muscle weakness and renal impairment (17) which is of interest since the two brothers with autistic features had these symptoms at times.

Unfortunately, we did not measure tartaric acid at the time these symptoms were present. The oral ingestion of as little as 12 grams of tartaric acid has been reported to cause a human fatality (18) while other studies indicate a much greater amount can be tolerated without causing toxicity (19-21). The guinea pig and pig are much more susceptible to renal damage by tartaric acid than the rat upon which much toxicological data have been gathered (22). A reassessment of tartaric acid toxicity may be warranted.

A compound with the same retention time and mass spectrum as arabinose was detected as present in high concentrations in the urine. Arabinose is present in a number of fruits but was not found as a major

component of 72 individuals with pentosuria (23). The carbohydrate alcohol arabitol is a carbohydrate produced by *Candida albicans* (24). Measurement of arabitol in human blood and in animal blood has been used as an indicator of the extent of *Candida* infection(25). These other studies employed GC or GC/MS of TMS derivatives as in this study. However, the retention time on GC and the electron impact mass spectra of these compounds are so similar that it is not clear whether arabitol and arabinose were differentiated in these other studies but they were definitely differentiated in our study.

Several possible explanations are available for the unusual metabolites present in these children with autistic features :

(1) These siblings with autistic features have a new genetic disease in which some abnormal metabolites (citramalic acid , tartaric acid) characteristic of yeast metabolism are excreted coincidentally but are not causally related to autism.

(2) These siblings may be infected with one or more yeasts and/or bacteria but this (these) infection(s) are secondary to immune deficiency which is common in autism (25).However, these metabolites are not causally related to autism.

(3) These siblings are infected with one or more microorganisms. The metabolites produced by these organisms are causally related to the disease through inhibition of mitochondrial Krebs cycle activity by citramalic, carboxycitric, 3-oxoglutaric, and tartaric acids and/or by interference in normal carbohydrate metabolism caused by high concentrations of arabinose. The severity of the symptoms of autism may be related to the amounts of abnormal

microbial metabolites produced as well as differences in the genetic ability to detoxify these microbial products.

Autism is a group of disorders with a wide spectrum of severity. It is unlikely that a single etiology may be found for all of these. However, the presence of abnormal metabolites in the urine of two siblings with autistic features raises the possibility that these may be casually related. Our investigation requires further analysis for similar urinary metabolites in a larger sample of autistic children and in pathological controls to determine if these abnormalities are found more commonly or are unique to these patients. Such studies are now in progress.

Figure Legends

FIGURE 1

A. Total ion current GC/MS chromatogram of the derivatized urine extract of autistic brother A. Peaks are identified as follows : A, glycollic; B,oxalic; C,3 -hydroxyisobutyric; D,urea; E, phosphoric; F,succinic; G,deoxytetronic; H,citramalic; I,undecanoic (internal standard); J,unidenitified; K,3-hydroxyphenylacetic; L,2-oxoglutaric; M,4-hydroxyphenylacetic;N, furandicarboxylic; O, furancarboxylglycine;P, taratric; Q, arabinose;R,aconitic; S,hippuric; T,citric; U,dihydroxyphenylpropionic; V,vanillylmandelic;W,3-indoleacetic; X, ascorbic; Y, citric analog; Z,uric;AA,unidentified;BB,4-hydroxyhippuric.

B. Total ion current GC/MS chromatogram of the derivatized urine extract of a typical normal child. Peaks are identified as follows:A, pyruvic; B,oxalic; C,urea; D,undecanoic; E,3-hydroxyphenylacetic;F,2-oxoglutaric; G,4-hydroxyphenylacetic;H, aconitic;I,J,hippuric;K,citric; L,vanillylmandelic;M,3-hydroxyhippuric.

FIGURE 2

- A. Mass spectrum of peak at 10.3 min from one of the urine samples from the brothers with autistic features.
- B. Mass spectrum of authentic citramalic acid.
- C. Mass spectrum of peak at 10.3 min from a different urine sample of one of the brothers with autistic features.

FIGURE 3

Proposed fragmentation pattern for 3-methylmalic acid

FIGURE 4

Comparison of citramalic acid (combined 2- and 3-methylmalic acid) concentrations in urine of brothers with autistic features with those of normal children.

FIGURE 5

- A. Mass spectrum of citric acid TMS derivative.
- B. Mass spectrum of isocitric acid TMS derivative
- C. Mass spectrum of tentatively identified citric acid analog TMS derivative.
- D. Mass spectrum of the same compound in C above which was derivatized with perdeuterated BSTFA.

FIGURE 6

Proposed fragmentation mechanism of citric acid, isocitric acid, and unknown TMS derivatives that yield 175 Da losses from the molecular ion.

FIGURE 7

Comparison of citric acid analog concentrations in urine samples from normal children and brothers with autistic features.

FIGURE 8

- A. Mass spectrum of 3-oxoglutaric acid TMS derivative from urine of

autistic child.

B. Mass spectrum of authentic 3-oxoglutaric acid TMS derivative.

C. Mass spectrum of 2-oxoglutaric acid TMS derivative.

FIGURE 9

Comparison of tartaric acid concentrations in urine samples from normal children and brothers with autistic features.

FIGURE 10

Mass spectrum of the phenylcarboxylic acid found in high concentration in urine samples of the brothers with autistic features.

FIGURE 11

Comparison of the concentration of the phenylcarboxylic acid in urine samples from normal children and brothers with autistic features.

FIGURE 12

Comparison of the mass spectrum of arabinose in the urine of the brothers with autistic features with that of authentic standard.

FIGURE 13

Comparison of the concentration of arabinose in urine samples from normal children and brothers with autistic features.

FIGURE 14

Structures of Krebs cycle analogs found in urine samples from patients with autistic features and normal Krebs cycle metabolites

FIGURE 15

Hypothetical biosynthetic route from the production of carboxycitric acid contrasted with known routes for citric and methylcitric acids.

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Table 1
Interpretation of
unknown compound

Mass loss from 596	Major ions with plain TMS	Major ions d ₂ TMS	Δ	TMS content	Interpretation
523	73	82	9	1 TMS	TMS
449	147	162	15	1 TMS 1 DMS	TMS-O-DMS
407	189	?	?	?	?
375	221	242	21	2 DMS 1 TMS	?
323	273	291	18	2 TMS	M-COOTMS - COOHTMS -TMSOH
249	347	371	24	2 TMS 1 DMS	M-CH ₃ -2C00TMS
221	375	399	24	2 TMS 1 DMS	M-CH ₃ -COOTMS -OTMS
207	389	416	27	3 TMS	M-COOTMS -TMSOH
175	421	457	36	4 TMS	M- -CH ₂ COOTMS -COO
133	463	496	33	3 TMS 1 DMS	M-CH ₃ -COOHTMS
105	491	524	33	3 TMS 1 DMS	M-CH ₃ -TMSOH
15	581	623	42	4 TMS 1 DMS	M-CH ₃

Table 2

Interpretation of mass spectra of peak at 18.86 minutes obtained with deuterated and nondeuterated TMS derivatives.

Loss from 390	Major ions with plain TMS	Major ions d ₂ TMS	Δ	TMS content	Interpretation
325	65	65	0	0	C ₅ H ₅
317	73	82	9	1	TMS
299	91	91	0	0	C ₇ H ₇
290	100	106	6	1DMS	DMS-O-CH=CH
277	113	122	9	1TMS	TMS-O-CH ₂ -CH ₂
243	147	162	15	1TMS 1DMS	TMS-O- DMS
235	155	164	9	1TMS	?
161	229	244	15	1TMS 1DMS	?
117	273	282	9	1TMS	M-COOTMS
91	299	317	18	2TMS	M-C ₇ -H ₇
53	337	355	18	2TMS	M-CH ₂ CH=CHCH
40	350	368	18	2TMS	M-CH ₂ CH=CH
15	375	390	15	1TMS 1DMS	M-CH ₃
0	390	408	18	2TMS	M