FEBS Letters 586 (2012) 956–961

FEBS Letters

journal homepage: www.FEBSLetters.org

Hypothesis

Metabolic differences underlying two distinct rat urinary phenotypes, a suggested role for gut microbial metabolism of phenylalanine and a possible connection to autism

T. Andrew Clayton

The Winston Churchill School, Hermitage Road, St. Johns, Woking, Surrey GU21 8TL, UK

ARTICLE INFO

Article history:
Received 2 November 2011
Revised 8 January 2012
Accepted 23 January 2012
Available online 1 February 2012

Edited by Judit Ovádi

Keywords:
Chlorogenic acid
Phenylalanine
Bacteria
Clostridia
Autism
Indolylacryloylglycine

ABSTRACT

A novel explanation is proposed for the metabolic differences underlying two distinct rat urinary compositional phenotypes i.e. that these may arise from differences in the gut microbially-mediated metabolism of phenylalanine. As part of this hypothesis, it is further suggested that elements of the mammalian gut microbiota may convert phenylalanine to cinnamic acid, either by means of an ammonia lyase-type reaction or by means of a three step route via phenylpyruvate and phenyllactate. The wider significance of such conversions is discussed with similar metabolism of tryptophan and subsequent glycine conjugation potentially explaining the origin of trans-indolylacryloylglycine, a postulated marker for autism.

1. Metabolite profiling

Metabolite profiling ('metabonomics'/'metabolomics') is a rapidly growing area of analytical science that has immense biomedical potential, with urine samples being particularly informative, convenient to obtain and analyse [1]. A particular strength of this still-developing analytical approach is that metabolite profiles are sensitive to a whole range of factors, both genomic and environmental, and, thereby, provide a multifactorial overview of a subject's status. In one general application of this 'systems biology' approach, the changes that are induced in endogenous metabolite profiles by stressors, such as drugs and toxins, are monitored and evaluated [1]. However, 'baseline' metabolite profiles are also highly informative and may, for instance, be indicative of gender, other genetic factors, dietary status or disease [1,2]. A great deal remains to be understood regarding the significance of inter-subject variation in baseline metabolite profiles and, to do so, such variation has to be correlated with known factors. One new approach, called 'pharmaco-metabonomic phenotyping', or simply 'pharmacometabonomics', has considerable potential to reveal the hidden significance of baseline metabolite profiles [3,4]. Thus, in a first study in humans [4], the presence of high levels of p-cresol sulfate in the urine has been associated with a lower residual sulfonation capacity and an important and hitherto-unrecognised effect of the gut microbiota on paracetamol (acetaminophen) metabolism has been demonstrated. That study provides an important insight into the impact of the gut microbiota on drug metabolism [5] whilst also serving as a reminder of the major role gut microbes play in dictating urinary composition [6]. The present report further discusses the impact of the gut microbiota on baseline urinary metabolite profiles and addresses the origins of two different rat urinary phenotypes.

2. The 'chlorogenic acid' phenotype and its origins

Rats are an important experimental model for many investigations and, in recent years, there have been repeated reports of two distinct rat urinary phenotypes, which are not attributable to dietary differences [7–10]. These phenotypes, which may be readily distinguished by ^{1}H NMR spectroscopy, differ in the relative amounts of hippurate (benzoyl glycine) and certain so-called 'chlorogenic acid metabolites'. Thus, the 'HIP' phenotype, which is generally regarded as the more typical and conventional phenotype [10,11], is characterised by a relatively high level of hippurate whilst the chlorogenic acid (CA) phenotype is characterised by a relatively low level of hippurate and by an increased amount of 3-(3-hydroxyphenyl)propionic acid (3-HPPA) [7–10]. Additionally, increased urinary amounts of two other metabolites have been

E-mail address: a.clayton@wcsc.org.uk

0014-5793/$36.00
doi:10.1016/j.febslet.2012.01.049

reported for the CA phenotype. Thus, Gavaghan et al. [8] reported increased levels of both 3-HPPA and 3-hydroxycinnamic acid (3-HCA), whilst Robosky et al. [10] reported increased levels of both 3-HPPA and 3-(4-hydroxyphenyl)propionic acid (4-HPPA).

Over time, the origin of these two distinct phenoptypes has been firmly attributed to differences in the gut microbiota, with rats of the CA phenotype being obtained from microbiologically-restricted environments and being convertible to the HIP phenotype by exposure to the normal laboratory environment or by cohabitation with HIP rats, a procedure which would be expected to lead to cross ingestion of faecal matter and of the associated microorganisms [7,10,11]. Thus, it has become clear that CA rats are in some way deficient in the gut microbes that are necessary to produce the HIP phenotype and, in one report, this deficiency has been attributed to the initiation of colonies from Schaedler altered microflora rats and to rigorous animal husbandry practices that prevent or delay subsequent microbiological contamination [11]. However, despite apparent acceptance that the observed phenotypic difference is associated with differences in the metabolism of dietary plant phenolics and that the observed 3-HPPA, 4-HPPA and 3-HCA metabolites are derived from chlorogenic acid [8,10,11], no clear explanation has been provided that details the exact metabolic deficiency involved in producing the CA phenotype.

In reviewing these earlier findings, the view was taken that the CA phenotype might, to some extent, resemble an in-born error of metabolism wherein a particular pathway is blocked or restricted and 'upstream' metabolites are directly observed or are diverted to different metabolic pathways. The work of Phipps et al. [7] shows that rats of the CA phenotype are still capable of conjugating benzoic acid with glycine to produce hippurate. Thus, it seemed appropriate to consider the possibility that, in the CA phenotype, a pathway that would otherwise lead to benzoic acid is restricted and that 3-HCA, 3-HPPA and 4-HPPA are upstream metabolites or are derived from such metabolites. Furthermore, in order for blockage of this pathway to lead to a clearly detectable decrease in urinary hippurate, it would need to provide a significant proportion of the benzoic acid utilised for urinary hippurate production in HIP phenotype rats. From this viewpoint and having seen a report that some human colonic bacteria can convert phenylalanine to benzoic acid in a multistep process that has phenylpropionic acid as an intermediate [12], it seemed possible that the origin of the CA/HIP phenotypic difference might lie in the metabolism of phenylalanine rather than in the metabolism of chlorogenic acid. This hypothesis would also fit with the assertion of Sakai et al. [13] that serum hippuric acid is derived primarily from aromatic amino acids by the metabolism of intestinal bacteria. At this point, it is worth noting that sodium benzoate is used as a preservative in some products consumed by humans but, in the absence of information to the contrary, it is assumed that a significant level of benzoate was not present in the diets of the rats used in the studies revealing the CA/HIP phenotypic difference. Furthermore, if, in the HIP phenotype, most of the observed hippuric acid had been derived from dietary benzoate, it would seem highly improbable that the CA phenotype could have been observed, bearing in mind the continued feasibility of benzoic acid–glycine conjugation in rats of that phenotype [7].

3. Relevant known and postulated metabolic pathways

Fig. 1 shows the microbially-mediated metabolism of phenylalanine to phenylacetic acid and of tyrosine to p-cresol [4,14]. These pathways show much commonality but also a notable divergence in that the presence of the p-hydroxy group in (tyrosine-derived) 4-hydroxyphenylacetic acid leads to loss of the acid group and

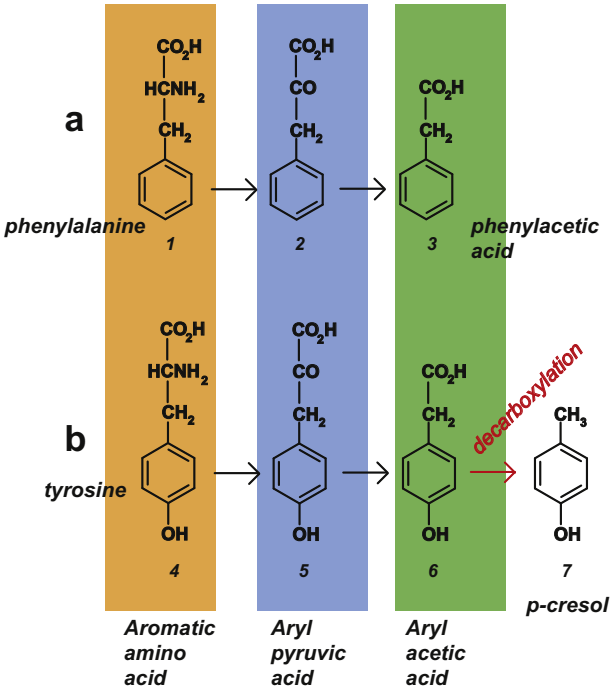

Fig. 1. The known microbially-mediated conversion, (a) of phenylalanine to phenylacetic acid and, (b) of tyrosine to p-cresol. Key to compounds: ***1***, phenylalanine; ***2***, phenylpyruvic acid; ***3***, phenylacetic acid; ***4***, tyrosine; ***5***, 4-hydroxyphenylpyruvic acid; ***6***, 4-hydroxyphenylacetic acid; ***7***, p-cresol. The structural representations provided are not intended to convey any stereochemical information. In humans, phenylacetic acid and p-cresol are further metabolised to phenylacetylglutamine and p-cresol sulfate, respectively.

the formation of p-cresol. However, as indicated in the report of Smith and Macfarlane [14], another bacterially-mediated metabolic possibility exists for these two aromatic amino acid substrates, phenylalanine and tyrosine, that seems particularly relevant to the present topic. Thus, Smith and Macfarlane indicate that aromatic amino acids ($R–CH_2–CH(NH_2)–CO_2H$) may be converted, via intermediates, to the corresponding aryl propionic acid ($R–CH_2–CH_2–CO_2H$). Evidence in support of such overall conversion, by whatever mechanism, is provided by the studies of Moss et al. [15], which show that phenylalanine may be converted to 3-phenylpropionic acid (PPA, hydrocinnamic acid) by certain species of *Clostridium*, with cinnamic acid (a *trans* alkene) being a possible intermediate in that conversion. Likewise, studies by Lambert and Moss [16] showed the production of 3-(4-hydroxyphenyl)propionic acid (4-HPPA) from tyrosine by *Peptostreptococcus anaerobius* and, in this case, it was suggested that there may have been an initial deamination to 4-hydroxycinnamic acid (4HCA; p-coumaric acid) and subsequent reduction to 4-HPPA. In plants, the production of cinnamic acid from phenylalanine is well known and is catalysed by phenylalanine ammonia lyase (PAL) [17,18]. It is also known that plants may further convert phenylalanine-derived cinnamic acid to benzoic acid with evidence supporting a β-oxidation type pathway [19]. However, whilst such reactions are best known in plants, there is now also evidence that similar events occur in some bacteria and fungi [18,20]. Thus, in *Penicillium brevicompactum*, it has been established that cinnamate is an intermediate in the conversion of l-phenylalanine to benzoate [21] whilst Moore et al. [22,23] report that the sedimentary bacterium *Streptomyces maritimus* produces benzoyl-CoA from phenylalanine in a plant-like manner that involves a PAL-mediated conversion of phenylalanine to cinnamic acid. Such pathways appear to provide a new but plausible explanation for the observed CA/HIP phenotypic

difference, which might, seemingly, be related more to the gut microbial metabolism of phenylalanine than to the gut microbial metabolism of dietary plant phenolics. Relevant postulated metabolic transformations are shown in Fig. 2.

As regards Fig. 2, the reactions of fatty acid β-oxidation could account for the conversion to benzoic acid of both cinnamic acid and 3-phenylpropionic acid, although the conversion of cinnamic acid to benzoic acid might also proceed by a somewhat different mechanism [19,24]. Blockage of those conversions would be expected to lead to an initial build up of both cinnamic and 3-phenylpropionic acids and, following such build up, aromatic hydroxylation, a typical phase 1 metabolic conversion, would readily explain the origin of the 3-HPPA and 3-HCA reported by Gavaghan et al. [8] and the origin of the 3-HPPA and 4-HPPA reported by Robosky et al. [10]. Such hydroxylation might potentially be achieved by elements of the gut microbiota [25]. However, aromatic hydroxylation is not, seemingly, a typical gut microbial conversion [26] and the involvement of the host's own cells in achieving this conversion seems more likely. In parallel with what is suggested here for phenylalanine, tyrosine would be expected to undergo an initial deamination to p-coumaric acid. However, as will be discussed below, tyrosine would not be expected to undergo exactly the same metabolic conversions as phenylalanine and the postulated divergence of phenylalanine and tyrosine metabolism in Fig. 2 parallels the known metabolic divergence shown in Fig. 1. The construction of Fig. 2 has been much influenced by the report of Scheline [26], which states that the microbially-mediated decarboxylation of phenolic acids is favoured by the presence of a p-hydroxyl group and has been observed with benzoic, phenylacetic and cinnamic acids but not with phenylpropionic acids. If there is sufficient opportunity for 4-HCA to undergo such microbially-mediated decarboxylation, these metabolic factors might account for the fact that, in the CA phenotype, both 3-HPPA and 4-HPPA have been observed along with 3-HCA but not 4-HCA. Furthermore, in Fig. 2, increased conversion of compound 2 to compound 3 would readily explain why Robosky et al. [10] found 3-HPPA and 4-HPPA in the CA phenotype in contrast to the 3-HCA and 3-HPPA reported by Gavaghan et al. [8]. The apparent absence of 2-position (*ortho*) hydroxylation is also potentially explicable, on the basis of steric hindrance. One significant implication of the proposed reaction scheme shown in Fig. 2 concerns the conversion of compounds 2 and 3 to benzoic acid, which might occur by the reactions of fatty acid β-oxidation. If, as suggested in Fig. 2, failure of these conversions underlies the origin of the CA phenotype, then it would appear that, in the HIP phenotype, such conversions must be achieved by the gut microbiota rather than by the host cells. However, another possibility is that, in producing the CA phenotype, competitive utilisation of phenylalanine simply reduces the availability of phenylalanine for benzoic acid production. However, whatever the cause of the reduced urinary hippuric acid excretion observed in the CA phenotype, there appears to be a clear possibility that, in that phenotype, phenylalanine is converted to cinnamic acid via a PAL-like deamination reaction with subsequent hydroxylation and reduction occurring. However, whilst the action of PAL, or similar, on phenylalanine presents a plausible route to cinnamic acid, such conversion might also be achieved according to the generalised scheme provided by Smith and Macfarlane [14] for the metabolism of aromatic amino acids

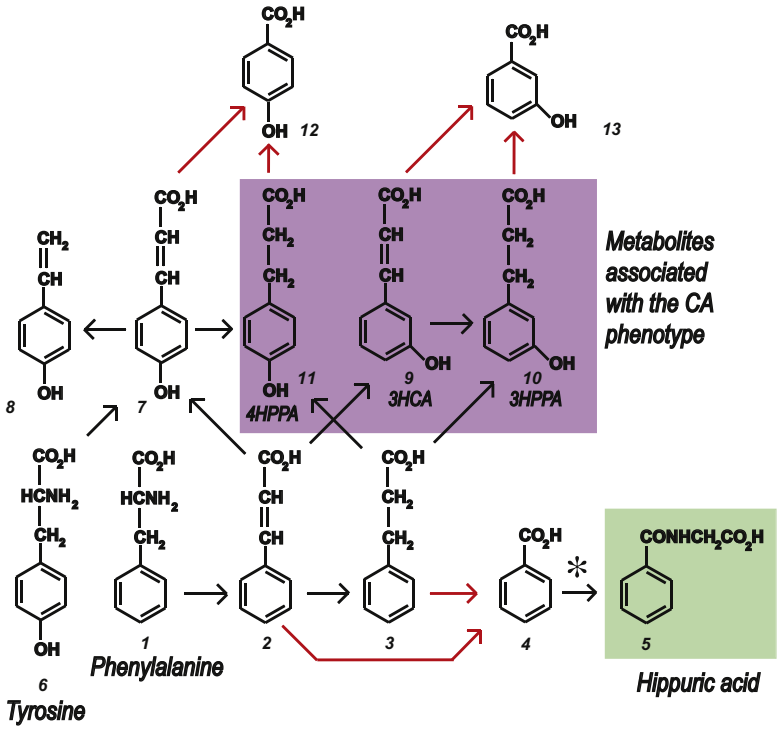

Fig. 2. Postulated metabolic pathways for phenylalanine and tyrosine. The red arrows indicate multi-step metabolic conversions resembling fatty acid β-oxidation, which might occur in the HIP phenotype but might not occur in the CA phenotype. The green box highlights hippuric acid, a high urinary level of which is observed in the HIP phenotype. The mauve box highlights three metabolites, higher urinary levels of which are associated with the CA phenotype. Key to compounds: ***1***, phenylalanine; ***2***, cinnamic acid; ***3***, 3-phenylpropionic acid (hydrocinnamic acid); ***4***, benzoic acid; ***5***, hippuric acid (benzoylglycine); ***6***, tyrosine; ***7***, 4-hydroxycinnamic acid (4HCA; p-coumaric acid); ***8***, 4-vinylphenol; ***9***, 3-hydroxycinnamic acid (3-HCA); ***10***, 3-(3-hydroxyphenyl)propionic acid (3-HPPA); ***11***, 3-(4-hydroxyphenyl)propionic acid (4-HPPA); ***12***, 4-hydroxybenzoic acid; ***13***, 3-hydroxybenzoic acid. The structural representations provided are not intended to convey any stereochemical information. The conversions of ***1***–***2*** and of ***6***–***7*** might proceed in a single step catalysed by some type of aromatic amino acid ammonia lyase (E.C. 4.3.1.23, E.C. 4.3.1.24, E.C. 4.3.1.25). Alternatively, these conversions might proceed according to the scheme of Smith and Macfarlane [14]; see Fig. 3. * The conversion of benzoic acid to hippuric acid is non-microbial and is performed by the host. The various hydroxylation reactions may also be non-microbial.

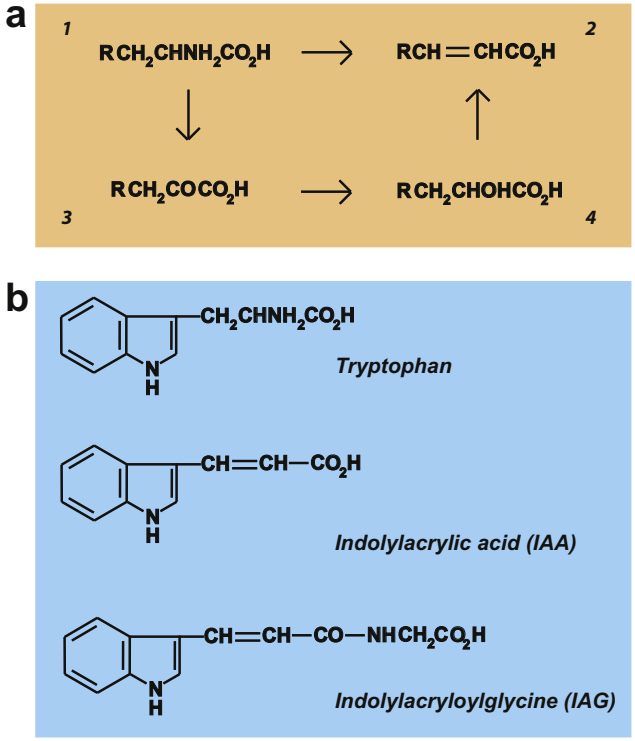

Fig. 3. (a) Two possible routes from aromatic amino acids to the corresponding *trans*-aryl alkenoic acids. ***1*** Designates an aromatic amino acid (phenylalanine, tyrosine or tryptophan), where R is the appropriate aryl moiety, ***2*** is the corresponding *trans*-aryl alkenoic acid, ***3*** is the corresponding aryl pyruvic acid and ***4*** is the corresponding aryl lactic acid. The structural representations provided are not intended to convey any stereochemical information and it remains to be established if the alkenoic acids produced by these two pathways would always have the trans configuration. The direct route from ***1*** to ***2*** requires the action of an appropriate ammonia lyase e.g. phenylalanine ammonia lyase. The other suggested possibility, an indirect route from ***1*** to ***2***, proceeds through ***3*** and ***4*** and follows the scheme of Smith and Macfarlane [14]. If compound ***1*** was tryptophan, compound ***2*** would be *trans*-3-indolylacrylic acid (IAA) and glycine conjugation of IAA would produce *trans*-indolylacryloylglycine (IAG), which has been proposed as a putative marker for autism. If compound ***1*** was phenylalanine, compound ***2*** would be cinnamic acid, which might potentially be metabolised to 3-(3-hydroxyphenyl)-3-hydroxypropionic acid [32] by meta hydroxylation and the addition of water across the double bond. If compound ***1*** was tyrosine, compound ***2*** would be 4-hydroxycinnamic acid, which is also known as p-coumaric acid. (b) The structures of tryptophan, indolylacrylic acid (IAA; 3-indoleacrylic acid) and indolylacryloylglycine (IAG) shown in a simplified style without any intention to convey stereochemical information.

(AAA) by human intestinal bacteria. In that scheme, the relevant metabolic route involves an initial conversion of the AAA to the corresponding aromatic pyruvic acid and subsequent conversion to the corresponding lactic acid before dehydration to an alkenoic acid. Reduction of the alkenoic acid was indicated to produce the corresponding propionic acid. Thus, whilst the respective conversions of phenylalanine and tyrosine to cinnamic acid and 4-hydroxycinnamic acid, shown in Fig. 2, might involve one-step ammonia lyase-type reactions, there is also a more established multi-step possibility for each of these conversions, as discussed by Smith and Macfarlane [14] and subsequently by Kim et al. [27]. These two different possibilities are shown in Fig. 3a.

4. Significance and a possible connection to autism

Metabolite profiling is proving to be an extremely powerful investigative approach. However, for the results of such studies to be interpreted correctly it is important to understand the factors controlling the nature of baseline metabolite profiles. Any abnormality in such profiles also raises a question regarding the status of the subjects studied and the validity and relevance of the experiments performed. However, understanding the origins of the CA/HIP phenotypic difference is not only important in relation to metabonomic/metabolomic studies in the rat and there is growing recognition of the potential importance of the gut microbiota in relation to disease and adverse drug reactions [5] with an abnormal gut microflora potentially increasing or decreasing susceptibility according to the nature of each process. Thus, the present considerations may have much wider significance and, for example, given the energy recovery value of fatty acid oxidation and the reported association between obesity and an altered gut microbiome [28], it is interesting to speculate that the CA phenotype might be associated with reduced energy recovery. However, most notably and clearly, the present considerations have potential relevance to the production of a postulated biomarker for autism, trans-indolylacryloylglycine (IAG) [29] with autistic subjects also having been associated with altered gut microbial populations [30]. Thus, the first metabolic steps shown in Fig. 2 for both phenylalanine and tyrosine are deaminations that involve, or equate to, the removal of ammonia. If the same metabolic transformation is applied to tryptophan, trans-3-indolylacrylic acid (IAA) would be produced and glycine conjugation of IAA would produce IAG. Notably, such deamination of tryptophan, if achieved via an ammonia lyase reaction, would serve to provide a clear and direct one-step route to IAA, in contrast to the more protracted and somewhat obscure scheme described by Marklova [31] (see Supplementary text). Making a further possible connection to autism, hydration of 3-HCA (compound 9 in Fig. 2) could potentially explain the formation of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid, increased urinary excretion of which has recently been reported in autistic and schizophrenic subjects [32]. Further still, Lis et al. [33] have reported elevated urinary levels of 4-hydroxyhippuric acid in autistic subjects and the immediate pre-conjugation precursor of that compound, 4-hydroxybenzoic acid, could potentially be derived from either phenylalanine or tyrosine (see Fig. 2) with further literature evidence confirming known microbial conversion of p-coumaric acid to 4-hydroxybenzoic acid [34,35]. Thus, whilst the validity of IAG as a marker for autism has been challenged [36], there are at least three possible markers for autism that might be derived from the relevant aromatic amino acid via an initial ammonia lyase reaction and only a very few subsequent metabolic steps. This high degree of metabolic proximity suggests some connection between autism and abnormal gut microbial metabolism of aromatic amino acids. It is also notable, in view of the present considerations and the known origin of human urinary phenyacetylglutamine (PAG) ([4] and Fig. 1a), that a recent metabonomic study of autistic children, their unaffected siblings and age-matched controls [37] indicated a trend towards decreased excretion of hippurate and PAG in the autistic group. This also suggests some connection between autism and abnormal gut microbial metabolism of phenylalanine whilst the well known condition phenylketonuria makes a clear connection between abnormal phenylalanine metabolism and severe neurological symptoms; children with untreated phenylketonuria often also having autism [38].

As far as this author is aware, it remains to be established if abnormal gut microbial metabolism of aromatic amino acids has any role whatsoever in the development of autism. However, it might perhaps be that, as previously suggested [4], gut bacterial production of p-cresol (Fig. 1) is a significant factor in regard to autism, with *Clostridium difficile* being one notable p-cresol producer [39]. Thus, through its known inhibitory effect on dopamine β-hydroxylase [40], p-cresol might directly affect the metabolism of dopamine, which is an important neurotransmitter. Furthermore, childhood autism has been linked to impaired sulfonation [41] and it has been clearly shown [4] that, through its own

sulfonation, gut bacterially-produced p-cresol can significantly reduce an individual's sulfonation capacity with sulfonation being hugely important for a variety of normal processes and structures, for xenobiotic excretion and for catecholamine handling [42]. Further still, through its inhibitory effect on dopamine β-hydroxylase and the likely consequent excess of dopamine, this author considers that p-cresol production might readily explain the origins of another possible urinary biomarker for autism, homovanillic acid [43], which is the normal end product of dopamine degradation [44]. In regard to the onset of autism during childhood, it is also notable that sulfonation is considered to be especially important during early human development with glucuronidation being under-developed in the neonate [45,46]. In view of these considerations, it is also particularly notable that direct experimental evidence of a link between p-cresol and autism has recently been obtained [47] (see Supplementary information).

5. Conclusions

The origins of the CA phenotype still require clarification. However, the ideas presented here appear to have some merit for explaining the observed CA/HIP phenotypic difference and the origin of the benzoic acid that goes on to become urinary hippurate. If the metabolites that characterise the CA phenotype are not solely or almost entirely derived from chlorogenic acid, then the term 'CA phenotype' would appear to be a confusing misnomer and, as a precautionary measure, it would seem safer to follow the example of Williams et al. [9] and to refer instead to m-HPPA (3-HPPA) excretors. However, whatever the precise origins of the HIP and CA phenotypes, a key message of the present considerations is the possibility that certain elements of the mammalian gut microbiota might have PAL or similar activity and that such activity could potentially explain the origins of various compounds that have been linked to autism (Fig. 3). Alternatively, the scheme of Smith and Macfarlane [14] shows that aryl alkenoic acids may be produced from aromatic amino acids by a multi-step pathway (Fig. 3). Conceptually, it is certainly to be expected that particular gut microbes and populations that provide unusual metabolic capacities might have a role in the aetiology of various human diseases as well as in the development of adverse drug reactions in subsets of the human population [5]. It is also to be expected that identifying such microbial species and populations and determining the relevant metabolic factors may require considerable research effort. However, from the present discussion and given the suggested link between autism and clostridia [48], it would seem sensible to investigate if any species of clostridia inhabiting the human gut are able to provide significant activity of any aromatic amino acid ammonia lyase. Possession of PAL, or similar, activity by elements of the clostridia would not be especially surprising given the other unusual metabolic features of these bacteria [49], some of which are notable toxin producers. Likewise, the present considerations suggest that it would be useful to perform a detailed survey, for each aromatic amino acid, to establish which of the clostridia inhabiting the human gut are able, like *Clostridium sporogenes* and phenylalanine [27], to metabolise that compound to the corresponding *trans*-aryl alkenoic acid according to the scheme of Smith and Macfarlane [14]. The justification for such investigations is to elucidate the potential origins of various compounds linked to autism. Conceivably, it might also be worthwhile to examine the fungal component of the gut microbiota for PAL activity. The justification here is that PAL occurs abundantly in yeast [20,50] and that there has been some suggestion of a link between yeasts and autism and of improvements in autistic patients following antifungal therapy. Thus, it is conceivable that fungal degradation of phenylalanine has some role in autism. It is also likely that, as with spore-forming clostridia, fungi such as *Candida* are able to take a much stronger hold in the gut when, for reasons such as extensive antibiotic treatment, the extent of normal gut bacterial colonisation is much reduced.

In summary, consideration of the 'chlorogenic acid' urinary phenotype seen in rats, suggests possible gut microbial production of cinnamic acid from phenylalanine. Such conversion might potentially occur by either of the mechanisms shown in Fig. 3a with the possible action of phenylalanine ammonia lyase representing a particularly novel and interesting possibility. With similar gut microbial conversion of phenylalanine, tyrosine and tryptophan potentially explaining the origins of three compounds linked to autism, and with clostridia and yeasts already suspected of involvement in its aetiology, a survey of their capacity for effecting such conversions may be warranted. It is also noted that the origins of two other compounds linked to autism and the diminished sulfonation capacity seen in autistic children might perhaps be explained by the action of certain gut microbes on tyrosine.

Acknowledgments

The author thanks Professor Glenn Gibson (University of Reading, UK) and Professor Jeremy Everett (University of Greenwich, UK) for comments received whilst drafting the manuscript.

Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2012.01.049.

References

[1] Lindon, J.C., Holmes, E., Bollard, M.E., Stanley, E.G. and Nicholson, J.K. (2004) Metabonomic technologies and their application in physiological monitoring, drug safety assessment and disease diagnosis. Biomarkers 9, 1–31.
[2] Bollard, M.E., Stanley, E.G., Lindon, J.C., Nicholson, J.K. and Holmes, E. (2005) NMR-based metabonomic approaches for evaluating physiological influences on biofluid composition. NMR Biomed. 18, 143–162.
[3] Clayton, T.A. et al. (2006) Pharmaco-metabonomic phenotyping and personalized drug treatment. Nature 440, 1073–1077.
[4] Clayton, T.A., Baker, D., Lindon, J.C., Everett, J.R. and Nicholson, J.K. (2009) Pharmacometabonomic identification of a significant host–microbiome metabolic interaction affecting human drug metabolism. PNAS 106, 14728–14733.
[5] Nicholson, J.K., Holmes, E. and Wilson, I.D. (2005) Gut microorganisms, mammalian metabolism and personalized health care. Nat. Rev. Microbiol. 3, 431–438.
[6] Nicholls, A.W., Mortishire-Smith, R.J. and Nicholson, J.K. (2003) NMR spectroscopic-based metabonomic studies of urinary metabolite variation in acclimatizing germ-free rats. Chem. Res. Toxicol. 16, 1395–1404.
[7] Phipps, A.N., Stewart, J., Wright, B. and Wilson, I.D. (1998) Effect of diet on the urinary excretion of hippuric acid and other dietary-derived aromatics in rat. A complex interaction between diet, gut microflora and substrate specificity. Xenobiotica 28, 527–537.
[8] Gavaghan, C.L. et al. (2001) Directly coupled high-performance liquid chromatography and nuclear magnetic resonance spectroscopic with chemometric studies on metabolic variation in Sprague-Dawley rats. Anal. Biochem. 291, 245–252.
[9] Williams, R.E., Eyton-Jones, H.W., Farnworth, M.J., Gallagher, R. and Provan, W.M. (2002) Effect of intestinal microflora on the urinary metabolic profile of rats: a ^{1}H-nuclear magnetic resonance spectroscopy study. Xenobiotica 32, 783–794.
[10] Robosky, L.C. et al. (2005) Metabonomic identification of two distinct phenotypes in Sprague-Dawley (Crl:CD(SD)) rats. Toxicol. Sci. 87, 277–284.
[11] Rohde, C.M. et al. (2007) Metabonomic evaluation of Schaedler altered microflora rats. Chem. Res. Toxicol. 20, 1388–1392.
[12] Macfarlane, S. and Macfarlane, G.T. (1995) Proteolysis and amino acid fermentation in: Human colonic bacteria: role in nutrition physiology and pathology (Gibson, G.R. and Macfarlane, G.T., Eds.), CRC Press, Boca Raton (Chapter 4).
[13] Sakai, T., Takadate, A. and Otagiri, M. (1995) Characterization of binding site of uremic toxins on human serum albumin. Biol. Pharm. Bull. 18, 1755–1761.
[14] Smith, E.A. and Macfarlane, G.T. (1997) Formation of phenolic and indolic compounds by anaerobic bacteria in the human large intestine. Microb. Ecol. 33, 180–188.
[15] Moss, C.W., Lambert, M.A. and Goldsmith, D.J. (1970) Production of hydrocinnamic acid by Clostridia. Appl. Microbiol. 19, 375–378.

[16] Lambert, M.A. and Moss, C.W. (1980) Production of p-hydroxyhydrocinnamic acid from tyrosine by *Peptostreptococcus anaerobius*. J. Clin. Microbiol. 12, 291–293.
[17] Camm, E.L. and Towers, G.H.N. (1973) Review article. Phenylalanine ammonia lyase. Phytochemistry 12, 961–973.
[18] Emiliani, G., Fondi, M., Fani, R. and Gribaldo, S. (2009) A horizontal gene transfer at the origin of phenylpropanoid metabolism: a key adaptation of plants to land. Biol. Direct 4, 7.
[19] Jarvis, A.P., Schaaf, O. and Oldham, N.J. (2000) 3-Hydroxy-3-phenylpropanoic acid is an intermediate in the biosynthesis of benzoic acid and salicylic acid but benzaldehyde is not. Planta 212, 119–126.
[20] MacDonald, M.J. and D'Cunha, G.B. (2007) A modern view of phenylalanine ammonia lyase. Biochem. Cell Biol. 85, 273–282.
[21] Campbell, I.M., Gallo, M.A., Jones, C.A., LaSitis, P.R. and Rosato, L.M. (1987) Role of cinnamate in benzoate production in *Penicillium brevicompactum*. Phytochemistry 26, 1413–1415.
[22] Moore, B.S. et al. (2002) Plant-like biosynthetic pathways in bacteria: from benzoic acid to chalcone. J. Nat. Prod. 65, 1956–1962.
[23] Xiang and Moore (2005) Biochemical characterization of a prokaryotic phenylalanine ammonia lyase. J. Bacteriol. 187, 4286–4289.
[24] French, C.J., Vance, C.P. and Towers, G.H.N. (1976) Conversion of p-coumaric acid to p-hydroxybenzoic acid by cell free extracts of potato tubers and *Polyporus hispidus*. Phytochemistry 15, 564–566.
[25] Huycke, M.M. and Moore, D.R. (2002) In vivo production of hydroxyl radical by *Enterococcus faecalis* colonizing the intestinal tract using aromatic hydroxylation. Free Radic. Biol. Med. 33, 818–826.
[26] Scheline, R.R. (1973) Metabolism of foreign compounds by gastrointestinal microorganisms. Pharmacol. Rev. 25, 451–523.
[27] Kim, J., Hetzel, M., Boiangiu, C.D. and Buckel, W. (2004) Dehydration of (*R*)-2-hydroxyacyl-CoA to enoyl-CoA in the fermentation of α-amino acids by anaerobic bacteria. FEMS Microbiol. Rev. 28, 455–468.
[28] Turnbaugh, P.J. et al. (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 444, 1027–1031.
[29] Bull, G. et al. (2003) Indolyl-3-acryloylglycine (IAG) is a putative diagnostic urinary marker for autism spectrum disorders. Med. Sci. Monit. 9, CR422–CR425.
[30] Parracho, H.M.R.T., Bingham, M.O., Gibson, G.R. and McCartney, A.L. (2005) Differences between the gut microflora of children with autistic spectrum disorders and that of healthy children. J. Med. Microbiol. 54, 987–991.
[31] Marklova, E. (1999) Where does indolylacrylic acid come from? Amino acids 17, 401–413.
[32] Shaw, W. (2010) 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. Nutr. Neurosci. 13, 135–143.
[33] Lis, A.W., McLaughlin, I., McLaughlin, R.K., Lis, E.W. and Stubbs, E.G. (1976) Profiles of ultraviolet-absorbing components of urine from autistic children, as obtained by high-resolution ion-exchange chromatography. Clin. Chem. 22, 1528–1532.
[34] Torres y Torres, J.L. and Rosazza, J.P.N. (2001) Microbial transformations of p-coumaric acid by *Bacillus megaterium* and *Curvularia lunata*. J. Nat. Prod. 64, 1408–1414.
[35] Sachan, A., Ghosh, S., Sen, S.K. and Mitra, A. (2006) Co-production of caffeic acid and p-hydroxybenzoic acid from p-coumaric acid by *Streptomyces caeruleus* MTCC 6638. Appl. Microbiol. Biotechnol. 71, 720–727.
[36] Wright, B. et al. (2005) Is the presence of urinary indolyl-3-acryloylglycine associated with autism spectrum disorder? Dev. Med. Child Neurol. 47, 190–192.
[37] Yap, I.K.S. et al. (2010) Urinary metabolic phenotyping differentiates children with autism from their unaffected siblings and age-matched controls. J. Proteome Res. 9, 2996–3004.
[38] Blau et al. (2010) Phenylketonuria. Lancet 376, 1417–1427.
[39] Sivsammye, G. and Sims, H.V. (1990) Presumptive identification of *Clostridium difficile* by detection of p-cresol in prepared peptone yeast glucose broth supplemented with p-hydroxyphenylacetic acid. J. Clin. Microbiol. 28, 1851–1853.
[40] Goodhart, P.J., DeWolf, W.E. and Kruse, L.I. (1987) Mechanism-based inactivation of dopamine beta-hydroxylase by p-cresol and related alkylphenols. Biochemistry 26, 2576–2583.
[41] Alberti, A., Pirrone, P., Elia, M., Waring, R.H. and Romano, C. (1999) Sulfation deficit in "low functioning" autistic children: a pilot study. Biol. Psychiatry 46, 420–424.
[42] Strott, C.A. (2002) Sulfonation and molecular action. Endocr. Rev. 23, 703–732.
[43] Kałuzna-Czaplińska, J., Socha, E. and Rynkowski, J. (2010) Determination of homovanillic acid and vanillylmandelic acid in urine of autistic children by gas chromatography/mass spectrometry. Med. Sci. Monit. 16, CR445–CR450.
[44] Eisenhofer, G., Kopin, I.J. and Goldstein, D.S. (2004) Catecholamine metabolism: a contemporary view with implications for physiology and medicine. Pharmacol. Rev. 56, 331–349.
[45] Coughtrie, M.W.H. (2002) Sulfation through the looking glass – recent advances in sulfotransferase research for the curious. Pharmacogenomics J. 2, 297–308.
[46] Arana, A., Morton, N.S. and Hansen, T.G. (2001) Treatment with paracetamol in infants. Acta Anaesthesiol. Scand. 45, 20–29.
[47] Altieri, L. et al. (2011) Urinary p-cresol is elevated in small children with severe autism spectrum disorder. Biomarkers 16, 252–260.
[48] Finegold, S.M. (2008) Therapy and epidemiology of autism – clostridial spores as key elements. Med. Hypotheses 70, 508–511.
[49] Elsden, S.R., Hilton, M.G. and Waller, J.M. (1976) The end products of the metabolism of aromatic amino acids by Clostridia. Arch. Microbiol. 107, 283–288.
[50] Hodgins, D.S. (1971) Yeast phenylalanine ammonia-lyase. J. Biol. Chem. 246, 2977–2985.