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Urinary and plasma organic acids and amino acids in chronic fatigue syndrome

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Abstract

Previous work by others have suggested the occurrence of one or more chemical or metabolic 'markers' for ME/CFS including specific amino acids and organic acids and a number of unidentified compounds (CFSUM1, CFSUM2). We have shown elsewhere that CFSUM1 is partially derivatised pyroglutamic acid and CFSUM2 partially derivatised serine and have suggested and demonstrated that the analytical methods used were unsuitable to identify or to accurately quantify urinary metabolites. We have now made a detailed analysis of plasma and urinary amino acids and of urinary organic acids from patients with ME/CFS and from three control groups. Fasting blood plasma and timed urine samples were obtained from 31 patients with CFS, 31 age and sex-matched healthy controls, 15 patients with depression and 22 patients with rheumatoid arthritis. Plasma and urinary amino acids and urinary organic acids were determined using established and validated methods and data compared by statistical analysis. None of the previously reported abnormalities in urinary amino acids or of organic acids could be confirmed. Results however provide some evidence in patients with ME/CFS for underlying inflammatory disease and for reduced intramuscular collagen with a lowered threshold for muscle micro-injury. These factors in combination may provide a basis for the fatigue and muscle pain that are the major symptoms in these patients.

Keywords: Urine; Blood plasma; CFSUM1; CFSUM2; Rheumatoid arthritis; Depression

1. Introduction

Chronic fatigue syndrome (CFS) [often linked with myalgic encephalomyelitis (ME)] is characterised by severely disabling fatigue, both mental and physical, combined with a variety of other symptoms [1–4] and with a relatively high prevalence in primary care

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patients [3]. The underlying pathophysiology involves elements of neuroendocrine, neurotransmitter and immune function, all of which individually may be subclinical and thus difficult to precisely identify or establish. The high prevalence and debilitating nature of the disorder has provoked extensive scientific and clinical studies in the search for a unifying physiological or metabolic cause. Despite this, the aetiology of the disease remains obscure.

McGregor, Dunstan and colleagues, using gas chromatography and mass spectrometry, reported increased levels of some urinary compounds in patients with ME/CFS, with reduced excretion in others, suggesting the possibility of one or more chemical or metabolic 'markers' for ME/CFS that may distinguish such patients from those with conditions with overlapping symptomatology [5,6]. These metabolites included amino acids (e.g. tyrosine, alanine, β-alanine, glutamic acid) and organic acids (e.g. succinic acid, aconitic acid) among those identifiable using the method employed in addition to various unidentified 'markers', especially CFSUM1 ('chronic fatigue syndrome urinary marker 1') to which high significance was ascribed. Levels of other metabolites, alanine, glutamic acid and another unidentified 'marker' CFSUM2, were reduced. Since their first reports, McGregor, Dunstan and colleagues have continued to evaluate patients with ME/CFS and other associated syndromes including chronic pain [7–11] using the same methodology to identify and quantify urinary metabolites, including other organic acid and amino acid species and to correlate the levels observed with the severity of the disorders and have also established a commercial base (BioScreen Ltd.; http://www.bioscreen.com.au/) for exploitation of metabolic profiling in such patients.

We have identified CFSUM1 as partially derivatised pyroglutamic acid and CFSUM2 as partially derivatised serine and have suggested and demonstrated that the analytical methods employed by McGregor et al [5] are unsuitable with which to identify or to accurately quantify urinary metabolites [12]. We have now made a detailed analysis of plasma and urinary amino acids and of urinary organic acids from patients with ME/CFS and from control subjects using established and validated methods, in order to examine whether any significant differences in these metabolites are observed.

2. Patients and materials

These studies were approved by the Wandsworth Local Research Ethics Committee and informed consent was obtained from all participants before involvement in the studies.

Patients with ME/CFS were defined according to the International Oxford and CDC criteria. The CDC criteria are shown in Table 1: patients with four or more of these symptoms were judged as meeting the criteria for CFS. None of the patients showed symptoms of depression. A total of 30 patients were recruited, 11 male and 19 female with an overall age range of 26–84 years (Table 2).

Fifteen patients with depression but without CFS and 22 patients with rheumatoid arthritis as a representative inflammatory disease were recruited as control patients (Table 2). All were free from concomitant diseases of liver, kidney and heart and were not receiving medications that would interfere with the analyses being undertaken.

Thirty healthy subjects were also recruited as a further control group, matched as closely as possible to age and sex distribution of the patients with CFS (Table 2) and also with their general lifestyle. Subjects on medications and who were smokers were excluded.

There were no significant differences in ages between the healthy controls, patients with ME/CFS or the patients with depression but patients with rheumatoid arthritis were significantly older than those in the other groups ($p<0.05$).

All patients and control subjects were provided with a questionnaire, a shortened form of that of Ray et al [13], to assess (A) their somatic symptoms and cognitive difficulties and (B) disability and recent course of illness. The scores for each section were combined to provide a Sickness Impact Profile Score (SIPS). The average SIPS for patients with ME/CFS

Table 1
CDC criteria of chronic fatigue syndrome

Impaired memory and concentration
Sore throat
Tender cervical or axillary lymph nodes
Muscle pain
Headaches of a new type or severity
Unrefreshing sleep
Post-exertional malaise for >24 h
Multi-joint pain

Table 2
Patient and control groups

Patient group	Number (M=male; F=female)	Age range (median)	Sickness impact profile score[a] (Median and range)
ME/CFS	11 M	26–63 (44)	65 (35–98)
	19 F	26–84 (45)	
Healthy	11 M	20–66 (45)	12 (0–34)
	19 F	26–79 (45)	
Depression	6 M	34–71 (48)	[b]
	9 F	24–59 (46)	
Rheumatoid arthritis	5 M	43–67 (57)	[b]
	17 F	39–65 (53)	

[a] Based upon a questionnaire (Ray et al [13]) to assess (A) their somatic symptoms and cognitive difficulties and (B) disability and recent course of illness. The scores for each section were combined to provide the Sickness Impact Profile Score (SIPS).

[b] No scores are given for the latter two groups of control patients because of small numbers and returns of incomplete questionnaires from several patients.

was 65, compared to 12 for the age and sex-matched healthy controls (Table 2).

Patients were asked to provide two samples of urine, the first sample passed in the morning for selective qualitative metabolite analysis [12] and a timed 6-h fasting collection for qualitative and quantitative analysis of amino acids and organic acids. On presentation to the clinic with the latter urine collection, and while the patients or subjects were still fasting, a sample of blood was collected into heparinised tubes kept on ice. The blood plasma and cells were separated as soon as possible after collection by centrifugation in a refrigerated centrifuge at 4 °C. Samples of urine and blood plasma were stored deep frozen at −20 °C until analysed.

3. Methods

Plasma and urine amino acids were determined using established methods as their phenylisothiocyanate (PITC) derivatives using reversed phase HPLC on a 250×4.6 mm Apex ODS II (3 μm) column (Jones Chromatography Ltd), 80 mM sodium acetate in 2.5% aqueous acetonitrile and aqueous acetonitrile-methanol mobile phases with detection at 254 nm. L-methionine sulphone, L-homoarginine and L-norleucine were used as internal standards and quantification was by comparison to standard mixtures of physiological amino acids taken through the same procedure. Quantification was validated through internal QC procedures and participation in the ERNDIM external quality assurance scheme for plasma amino acids [The European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited Metabolic disorders]. Urinary levels were expressed as a ratio to the urinary creatinine concentration (mmol/mol creatinine).

Urinary creatinine was assayed using a modified Jaffé method.

Urinary organic acids were determined using an established method utilising ethyl acetate extraction from acidified and sodium chloride-saturated urine after stabilisation of oxo acids using ethoxylamine hydrochloride and subsequent drying under a stream of dry nitrogen. *n*-Tetracosane and *n*-hexacosane internal standards (10 μL of a 200 g/L mixture in *n*-heptane) were added to the dry residue which was then derivatised by trimethylsilylation with 200 μL N,O-bis (trimethylsilyl)-trifluoroacetamide (BSTFA) in the presence of 50 μL of dry pyridine. The trimethylsilyl (TMS) and TMS-ethoxime derivatives were separated using programmed temperature capillary gas chromatography on a HP-1MS column (Agilent 25 m × 0.2 mm i.d., film thickness 0.33 μm) in a Hewlett-Packard 5980 gas chromatograph coupled to a HP 5970 mass selective detector. Compounds were identified from their electron impact (EI) mass spectra by comparison to spectra in private and commercial mass spectral libraries. Identified organic acids were quantified using response factors obtained using authentic standards taken through the same procedure; quantification was validated through the ERNDIM external quality control scheme for quantitative organic acids. Organic acid levels were determined from peak areas in the total ion chromatograms with the exception of 3-hydroxy-*n*-butyric, pyroglutamic and 5-hydroxyindoleacetic acids that were determined from the extracted ion chromatograms of their respective characteristic ions at m/z 117, 156 and 290 respectively. This eliminated the effects of co-eluting compounds that would compromise the accurate quantification of these organic acids. Results are expressed as ratios to the urinary creatinine concentration in mmol/mol creatinine.

Results were tabulated in Excel (Microsoft), and analysed using Analyse-It for Excel (Analyse-it Soft-

ware Ltd). Data were expressed as mean ± S.D. or range as appropriate, following a test for the normality of distribution. Differences between healthy subjects and patient groups were tested using unpaired independent *t*-tests and Mann–Whitney *U* tests dependent upon the normality of data distribution; $p<0.05$ was taken as indicating significance between means. Differences between groups were also tested using a 1-way analysis of variance with the healthy subjects as the control group. Where relatively few results were available or a number of values below the detection limits were observed, data are presented as ranges or 95% confidence limits.

4. Results

Table 3 shows plasma levels (μmol/L) of 26 amino acids for the four study groups. Patients with ME/CFS showed significantly lower concentrations of plasma taurine ($p<0.01$), histidine ($p<0.001$), tyrosine ($p<0.01$) and α-amino-*n*-butyric acid ($p<0.05$) in comparison to the healthy control group. Patients with depression showed significantly lower plasma serine ($p<0.01$), histidine ($p<0.05$) and α-amino-*n*-butyric acid ($p<0.01$) in comparison to healthy control subjects. Patients with rheumatoid arthritis showed a greater number of plasma amino acid concentrations that were significantly different to those in healthy control subjects, with significantly lower glycine ($p<0.05$), serine ($p<0.05$), histidine ($p<0.01$), asparagine ($p<0.01$), α-amino-*n*-butyric acid ($p<0.05$) and methionine ($p<0.01$) and significantly higher plasma glutamate ($p<0.01$) and phenylalanine ($p<0.05$).

Table 4 shows urinary levels, expressed in mmol/mol creatinine, of the same 26 amino acids for the four study groups. Patients with ME/CFS showed

Table 3
Plasma amino acids

Amino acid	Healthy controls	ME/CFS patients	Patients with depression	Patients with rheumatoid arthritis
β-alanine	<2–3	<2–3	<2	<2
Aspartate	<2–5	<2–6	<2–6	<2–8
Glutamate	26.9 ± 10.0	26.5 ± 13.2	35.4 ± 16.5	**40.3 ± 19.1**
Asparagine	57.4 ± 10.0	57.3 ± 15.2	57.3 ± 9.0	**50.4 ± 8.2**
Hydroxyproline	6.8 ± 2.6	6.1 ± 2.7	5.8 ± 2.0	7.5 ± 2.6
Serine	91.1 ± 17.4	83.5 ± 15.9	**77.1 ± 14.8**	**80.3 ± 19.5**
Glycine	226.4 ± 59.9	234.9 ± 66.1	226.7 ± 97.8	**187.6 ± 42.2**
Glutamine	470.9 ± 95.3	461.6 ± 89.8	458.1 ± 85.8	447.1 ± 90.0
Taurine	54.9 ± 22.3	**42.3 ± 10.6**	42.6 ± 5.1	54.3 ± 14.3
Histidine	70.4 ± 21.8	**52.4 ± 11.9**	**52.9 ± 9.6**	**54.0 ± 15.2**
Citrulline	(18–67)	(11–39)	(22–52)	(15–32)
Threonine	(50–263)	(68–232)	(79–198)	(76–332)
Alanine	287.5 ± 93.7	269.1 ± 65.7	308.4 ± 44.7	291.1 ± 102.3
Arginine	70.1 ± 24.1	72.3 ± 14.7	72.4 ± 23.6	72.8 ± 16.4
Proline	147.6 ± 48.4	142.9 ± 48.4	181.7 ± 68.4	163.5 ± 48.0
α-aminobutyric	20.5 ± 10.7	**14.8 ± 5.9**	**12.9 ± 5.1**	**14.7 ± 7.8**
Tyrosine	55.4 ± 12.8	**44.9 ± 12.1**	59.7 ± 16.3	63.0 ± 17.2
Valine	176.0 ± 38.3	173.3 ± 32.0	196.9 ± 38.8	188.8 ± 45.7
Methionine	19.6 ± 4.3	18.8 ± 3.5	18.1 ± 2.9	**16.8 ± 2.3**
Cystine	14.5 ± 9.2	17.9 ± 6.1	19.5 ± 12.3	19.1 ± 8.2
Isoleucine	57.8 ± 12.9	55.0 ± 15.1	63.4 ± 13.6	61.8 ± 21.3
Leucine	108.5 ± 21.5	105.2 ± 25.9	106.8 ± 16.8	103.8 ± 23.6
β-amino-isobutyric	<2–5	<2	<2	<2
Phenylalanine	50.2 ± 8.1	48.6 ± 8.5	53.3 ± 9.1	**56.3 ± 10.9**
Ornithine	61.3 ± 16.8	67.9 ± 15.5	62.9 ± 18.6	61.1 ± 14.3
Lysine	160.8 ± 30.4	160.8 ± 32.6	153.1 ± 28.0	151.5 ± 33.7

[Expressed as μmol/L; Mean ± S.D. or (range) if relatively few values for any group].
Patient data that are significantly different from healthy controls are in bold.

significantly lower levels of several urinary amino acids in comparison to levels in healthy control subjects [β-alanine, $p<0.05$; hydroxyproline, $p<0.0001$; histidine, $p=0.05$; methionine, $p<0.01$; cystine, $p<0.01$; phenylalanine, $p<0.01$] with correlation only with low plasma histidine concentrations. Patients with rheumatoid arthritis also showed several urinary amino acid levels that were significantly different from those in healthy control subjects with significantly lower levels of methionine ($p<0.01$), histidine ($p<0.05$) and glycine ($p<0.05$) but with significantly higher levels of glutamate ($p<0.05$), hydroxyproline ($p<0.01$), valine ($p<0.01$) and isoleucine ($p<0.05$). In this group there was correlation with low plasma methionine, histidine, and glycine and with high plasma glutamate; phenylalanine levels were also higher in rheumatoid patients but not significantly different from the levels in healthy control subjects. In contrast, patients with depression showed significant differences from healthy control subjects only for β-alanine levels that were significantly lower ($p<0.01$).

Table 5 shows the urinary levels, expressed in mmol/mol creatinine, for 16 organic acids for the four study groups. None of the groups showed any qualitative differences in urinary organic acid patterns and there were no obvious abnormalities or abnormal organic acids present. The number of organic acids in regularly observed in the urine of adults is relatively small, with wider quantitative variation than amino acids, but there were relatively few significant differences between the groups. All three patient groups showed significantly increased urinary 4-hydroxyphenylacetic acid levels in comparison to healthy control subjects (all with $p<0.05$). Patients with depression and with rheumatoid arthritis showed significantly higher levels of aconitic acid ($p<0.0001$ and $p<0.05$ respectively) and patients with rheumatoid

Table 4
Urine amino acids

Amino acid	Healthy controls	ME/CFS patients	Patients with depression	Patients with rheumatoid arthritis
β-alanine	1.27 ± 0.90	**0.82 ± 0.55**	**0.55 ± 0.46**	1.32 ± 1.14
Aspartate	5.20 ± 6.15	2.70 ± 3.00	3.05 ± 3.14	3.75 ± 5.08
Glutamate	7.89 ± 3.89	8.19 ± 2.06	9.23 ± 2.57	**10.41 ± 3.46**
Asparagine	10.52 ± 3.85	12.36 ± 6.41	13.12 ± 5.23	8.68 ± 4.63
Hydroxyproline	1.37 ± 0.78	**0.56 ± 0.54**	1.18 ± 0.54	**2.32 ± 1.48**
Serine	27.38 ± 9.54	26.95 ± 10.66	22.56 ± 8.50	22.85 ± 9.24
Glycine	136.48 ± 77.27	137.56 ± 64.38	138.52 ± 72.98	**97.87 ± 47.87**
Glutamine	33.32 ± 10.78	32.79 ± 12.46	29.97 ± 10.60	27.80 ± 10.41
Taurine	36.07 ± 29.16	46.39 ± 33.11	28.62 ± 27.26	56.68 ± 47.97
Histidine	60.75 ± 27.45	**46.12 ± 28.20**	49.15 ± 27.80	**43.25 ± 20.18**
Citrulline	0.24 ± 0.23	0.28 ± 0.16	0.68 ± 0.73	0.35 ± 0.21
Threonine	(5.17–24.59)	(10.53–13.98)	(–)	(4.41–25.84)
Alanine	19.46 ± 5.32	21.14 ± 9.23	22.39 ± 6.41	22.97 ± 13.74
Arginine	1.96 ± 1.77	1.23 ± 1.18	1.29 ± 0.89	2.63 ± 3.14
Proline	3.06 ± 1.87	3.77 ± 3.23	2.77 ± 1.35	3.30 ± 3.06
α-aminobutyric acid	1.37 ± 1.05	1.40 ± 0.75	1.56 ± 1.24	1.33 ± 1.14
Tyrosine	7.41 ± 2.30	6.35 ± 4.38	7.45 ± 2.21	8.39 ± 4.23
Valine	3.15 ± 1.49	3.91 ± 1.62	3.73 ± 1.86	**5.50 ± 4.32**
Methionine	1.70 ± 1.01	**1.04 ± 0.48**	1.49 ± 1.11	**0.80 ± 0.33**
Cystine	2.61 ± 1.42	**1.61 ± 1.09**	3.19 ± 1.52	2.91 ± 1.43
Isoleucine	3.55 ± 1.85	4.53 ± 2.40	4.69 ± 2.77	**4.98 ± 3.05**
Leucine	2.43 ± 0.77	2.02 ± 0.97	2.55 ± 1.06	2.80 ± 1.56
β-amino-isobutyric acid	6.81 ± 2.82	8.35 ± 7.05	6.07 ± 4.26	8.24 ± 9.61
Phenylalanine	4.50 ± 1.15	**3.71 ± 1.12**	4.13 ± 1.18	5.14 ± 2.64
Ornithine	4.11 ± 1.37	3.91 ± 1.72	3.44 ± 1.02	3.87 ± 1.50
Lysine	10.28 ± 5.34	11.59 ± 6.40	9.97 ± 3.73	10.60 ± 5.67

[Expressed as mmol/mol creatinine; Mean ± S.D. or (95% confidence limits) if relatively few values for any group].
Patient data that are significantly different from healthy controls are in bold.

Table 5
Urinary organic acids

Organic acid	Healthy controls	ME/CFS patients	Patients with depression	Patients with rheumatoid arthritis
Lactic	12.3 ± 6.2	13.3 ± 6.9	12.6 ± 11.4	14.4 ± 11.0
Succinic	5.6 ± 3.8	7.0 ± 4.1	6.8 ± 5.2	**3.5 ± 2.0**
Fumaric	(<0.1–0.4)	(<0.1–1.6)	(0.1–0.9)	(0.1–0.5)
2-oxoglutaric	(2.2–8.2)	(8.2–16.3)	(7.9–43.0)	(10.7–21.6)
Aconitic	13.9 ± 5.0	14.4 ± 10.4	**23.5 ± 7.0**	**20.2 ± 12.5**
Isocitric	22.0 ± 13.8	20.5 ± 9.5	24.6 ± 9.8	24.6 ± 10.4
Citric	217.8 ± 90.0	197.7 ± 91.0	203.1 ± 73.5	197.3 ± 118.3
3-hydroxy-*n*-butyric	1.4 ± 1.3	2.0 ± 3.5	1.0 ± 1.1	1.7 ± 3.7
Adipic	0.4 ± 0.6 (0.2–0.7)	0.5 ± 0.5 (0.4–0.7)	1.1 ± 1.1 (0.5–1.7)	0.9 ± 1.5 (0.2–1.5)
Suberic	(0–0.6)	(0–1.2)	(0–0.6)	(0–1.1)
4-hydroxybenzoic	1.8 ± 1.8	2.2 ± 1.5	1.5 ± 0.9	2.4 ± 1.6
4-hydroxyphenylacetic	7.4 ± 4.4	**11.2 ± 7.1**	**11.1 ± 6.7**	**12.2 ± 12.3**
Hippuric	298.5 ± 276.8	199.8 ± 166.7	405.4 ± 247.2	228.5 ± 197.7
Pyroglutamic	14.0 ± 7.2	12.0 ± 5.8	15.1 ± 6.9	17.9 ± 7.5
Vanilmandelic	1.2 ± 0.5	1.3 ± 0.1	1.2 ± 0.6	1.7 ± 1.2
5-hydroxyindoleacetic	1.1 ± 0.5	1.4 ± 0.8	1.4 ± 0.5	1.3 ± 0.3

[Expressed as mmol/mol creatinine; Mean ± S.D. and/or (95% confidence limits) if relatively few values for any group or if there were a number of undetectable (<0.1) values].
Patient data that are significantly different from healthy controls are in bold.

arthritis showed significantly lower levels of succinic acid, in comparison to those in healthy control subjects ($p<0.05$).

Urinary data are based upon the urinary creatinine output of the subjects studied. Urinary creatinine reflects the mean muscle mass of the subjects and because the latter might be reduced in patients with CFS and others because of reduced physical activity, a comparison of urinary creatinine concentrations (expressed as mmol/L; data not shown) was made and showed no significant differences between any of the groups studied.

5. Discussion and conclusions

Urinary amino acids showed more differences than plasma amino acids from those in healthy control subjects for both the ME/CFS and rheumatoid arthritis patients. Both patient groups showed low urinary histidine levels, correlated with low plasma histidine concentrations. Low histidine plasma levels have been observed previously in patients with active adult rheumatoid arthritis, correlating with the activity of the disease [14] and this may suggest a similar aetiology (active inflammatory disease) in patients with ME/CFS.

ME/CFS patients showed a highly significant reduction in urinary hydroxyproline levels in comparison to healthy control subjects and also in comparison to patients with depression ($p<0.01$) and patients with rheumatoid arthritis ($p<0.0001$). Reduced urinary hydroxyproline has been reported in patients with fibromyalgia [15]; these patients also have significantly lower concentrations of hydroxyproline in perimysium and endomysium of muscle [16], consistent with reduced intramuscular collagen, that may lower the threshold for muscle micro-injury and resulting in non-specific signs of muscle pathology. The present observations may reflect a similar basis in patients with ME/CFS that, in combination with active inflammatory disease, may provide a basis for the fatigue and muscle pain that are major symptoms in patients with ME/CFS.

Patients with ME/CFS also showed significantly low plasma concentrations of taurine, tyrosine and α-amino-*n*-butyric acid, not correlated with similar reductions in urinary levels of these amino acids where, in contrast, significantly reduced levels of methionine, cystine and phenylalanine were observed. All these amino acids are metabolically unrelated and their levels in both plasma and urine may be influenced to some extent by diet, exercise, renal function and other factors [17]. With

no clear pattern to, or relationship within, the reductions observed, their metabolic and clinical significance is unclear.

It is noteworthy that plasma and urinary serine concentrations were not significantly different in patients with ME/CFS when compared to those in healthy control subjects. The origin of the putative urinary 'marker' CFSUM2, originally suggested to be important by McGregor and co-workers [5,6], has been shown to be partially derivatised serine [12] and reduced urinary serine levels have been associated by these workers and their co-researchers with ME/CFS. The close similarity in the present work of urinary serine levels in all four groups, despite significantly reduced plasma concentrations in patients with depression and with rheumatoid arthritis, confirms these earlier observations on patients with ME/CFS were artefactual and caused by the non-standard methodology employed [12].

Patients with rheumatoid arthritis showed the largest number of amino acids with significantly different levels to those in healthy control subjects, again with a range of metabolically unrelated amino acids affected. Histidine and serine have been referred to above; the significance of low plasma and urinary glycine and methionine is unclear; increased urinary hydroxyproline may indicate slightly increased collagen turnover or bone resorption in these patients; reduced plasma asparagine has been reported in patients with leukaemia [17,18] and rheumatoid arthritis may be associated with leukocytosis, thus a similar aetiology may underlie this observation. Plasma and urinary glutamate were higher in patients with rheumatoid arthritis than healthy controls; increased plasma glutamate has been reported in patients with primary gout [19], hyperuricaemia is associated with leukocytosis and again a similar aetiology (chronic inflammatory disease and leukocytosis) may be the underlying cause. Plasma phenylalanine was also significantly increased in comparison to concentrations in healthy control subjects but the concentrations observed are within the reference ranges for this amino acid and are clinically insignificant in comparison to those seen in the hyperphenylalaninaemias.

Both ME/CFS patients and those with depression showed significantly lower levels of urinary β-alanine in comparison to healthy control subjects. This is in contrast to McGregor et al [5] who reported increased levels of β-alanine in patients with ME/CFS and ascribed a positive symptom incidence correlation with this amino acid. The clinical and pathological relevance of a reduced urinary β-alanine in these patients remains unclear although in the absence of measurable amounts in blood plasma, a renal origin is possible, possibly from β-alanyl peptides.

Urinary organic acids were generally unremarkable. Table 5 shows the urinary organic acids grouped according to their metabolic associations and essentially show that there were no clinically or pathologically important variations in the metabolism of pyruvate (lactate), the tricarboxylic acid cycle, ketone body production (3-hydroxy-*n*-butyric acid) or β-oxidation of fatty acids (adipic and suberic acids), in the γ-glutamyl cycle (pyroglutamic acid) or neurotransmitter metabolism. 4-hydroxyphenylacetic acid levels were significantly higher in the three patient groups and this may reflect slightly increased gut metabolism of tyrosine secondary to bacterial overgrowth, although the levels observed are all well below those observed in children and infants with proven bacterial overgrowth syndromes [20]. Hippuric acid levels are also influenced by gut bacterial amino acid metabolism but also, and more importantly, by ingestion of benzoic acid containing foodstuffs; that no differences were observed here between the groups studied suggests dietary influences were similar. The apparently reduced excretion of 2-oxoglutaric acid by patients with ME/CFS is a reflection of a relatively large number of undetectable values with a scatter of other levels overlapping considerably those in the other groups studied. Urinary 2-oxoglutaric acid may be influenced by a variety of factors including urinary tract bacterial activity [21] and the observations made here are not considered to be of any pathological importance. In the same way, the slight but significant increase in urinary aconitic acid levels in patients with depression and rheumatoid arthritis are not believed to be of any clinical or pathological significance.

Most importantly from the perspective of this study, none of the abnormalities in urinary organic acid excretion reported by McGregor et al [5,6] could be confirmed in the present work with use of established and externally validated methods and the

results reported by these workers may be ascribed to methodological differences and possible artefact formation [12]. In particular, the urinary levels of pyroglutamic acid, which has been shown to be the (artefactual) origin of the putative urinary marker CFSUM1 of McGregor and co-workers [12], show no significant differences between the groups studied. This confirms that pyroglutamic acid (and, by association, CFSUM1), which may be of primarily dietary origin in the adult without proven defects in the γ-glutamyl cycle [21], has no significance in the aetiology of ME/CFS.

In conclusion, the present work has provided definitive data on the major plasma and urinary amino acids and urinary organic acids. None of the previously reported abnormalities in urinary amino acids or of organic acids [5,6] could be confirmed and many of the abnormalities reported may be ascribed to artefacts introduced by their use of non-standard methods [12]. Comparison between the groups studied (patients with ME/CFS, patients with depression, patients with rheumatoid arthritis and healthy control subjects) has shown some evidence for underlying inflammatory disease in patients with ME/CFS (histidine) and although other manifestations of this seen in patients with rheumatoid arthritis (asparagine and glutamate) were not evident in these patients, the exact mechanisms involved may differ and warrant further investigation. The highly significant reduction in urinary hydroxyproline in patients with ME/CFS suggests, by analogy to similar observations in patients with fibromyalgia, reduced intramuscular collagen that may lower the threshold for muscle micro-injury and result in non-specific signs of muscle pathology. The present observations may reflect a similar basis in patients with ME/CFS. A reduced threshold for muscle micro-injury in combination with active inflammatory disease may provide a basis for the fatigue and muscle pain that are the major symptoms in patients with ME/CFS and this may also warrant further investigation.

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