Relative importance of formiminoglutamic and urocanic acid excretion after a histidine load

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SYNOPSIS A thin-layer chromatographic method for the simultaneous assessment of formiminoglutamic (Figlu) and urocanic acid excretion after loading with 15 g. histidine has been applied to 15 control subjects and to 49 selected patients. Upper limits of normal excretion were determined for urocanic acid and Figlu alone (15 mg. and 30 mg./8 hr. respectively), and for the combined metabolites (40 mg./8 hr.). Of the 49 patients studied, 27 excreted total metabolites above 40 mg./8 hr.; of these 19 would have been revealed by their abnormal excretion of Figlu and 22 by their abnormal excretion of urocanic acid. Of 74 tests in the 64 subjects, urocanic acid was present in all but 27% of the tests, and in 29.8% of the tests urocanic acid was in excess of the amount of Figlu excreted. In six normal and six abnormal tests urocanic acid was the sole metabolite present. These results re-emphasize the value of estimating both histidine metabolites in tests for folate deficiency in man, and underline the greater relative importance of urocanic acid.

The metabolic fate of L-histidine, summarized in Fig. 1, has been the subject of renewed interest because of the realization that some stages of its breakdown to glutamic acid are folate dependent (Bakerman, Silverman, and Daft, 1951; Borek and Waelsch, 1953; Tabor, Silverman, Mehler, Daft, and Bauer, 1953; Seegmiller, Silverman, Tabor, and Mehler, 1954). This interest has been further enhanced by the fact that the amount metabolized to glutamic acid can be augmented by suitable histidine loading (Tabor *et al.*, 1953; Luhby, Cooperman, and Teller, 1959a and b; Spray and

Witts, 1959), and that the intermediates of this metabolic pathway are readily accessible for examination in the urine.

In the absence of a competent folic acid coenzyme to act as an acceptor for the formimino group, formiminoglutamic acid (Figlu, Fig. 2) accumulates and is excreted in the urine. Several workers have reported significant increases in the urinary excretion of this metabolite in nutritional folic acid deficiency and in a variety of diseased states where factors other than dietary deficiency of folic acid operate to produce what might be described as 'conditioned

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FIG. 1. Metabolic fate of L-histidine

FIG. 2. Breakdown of histidine to glutamic acid

folate deficiency' and these have been well reviewed recently by Luhby and Cooperman (1964). The suggested conditioning factors have included malabsorption, increased demand and increased utilization of folic acid, interference with the proper functioning of the folate coenzymes due to vitamin B₁₂ deficiency, antagonism from synthetic therapeutic substances and even hypothetical naturally occurring folic acid antagonists, defective hepatic storage, and deficiency of the enzyme Figlu transferase, an apoenzyme in the liver which acts with tetrahydrofolic acid in the transfer of the formimino group (Fig. 2). Estimation of urinary Figlu, with or without histidine loading, is widely regarded as a useful and sensitive biochemical index of folate deficiency.

However, Acheson, Paul, and Tomlinson (1958) identified a precursor of Figlu, urocanic acid, in small amounts in normal urine, and in the same year Baldrige (1958) demonstrated a decrease in liver urocanase activity in folate-deficient rats. More recently, Bennett and Chanarin (1962) have demonstrated the presence of urocanic acid on histidine loading in 88% of a large number of patients with a variety of clinical disorders, including megaloblastic anaemia. In 79 of the 186 samples, urocanic acid exceeded the amounts of Figlu excreted and 10 patients excreted only urocanic acid. Inhibition of urocanase by high tissue Figlu levels with subsequent build-up of urocanic acid was considered as a possible mechanism. Reactivation of urocanase by the rising tissue levels of urocanic acid would, in turn, result in the reappearance of Figlu in the urine. Chanarin (1963) has since described a patient with megaloblastic anaemia showing an alternating pattern of Figlu and urocanic acid excretion on serial testing, as would be expected on this hypothesis.

Despite this important work, histidine load tests continue to be assessed widely on the basis of Figlu excretion only. The main reason for this is the technical difficulty of estimating Figlu and urocanic acid simultaneously. The spectrophotometric modification of the enzymatic assay developed by Chanarin and Bennett (1962) is tedious and time consuming, and the crude liver enzyme preparation used contains a variety of enzymes, including histidase and urocanase, both of which are potentially liable to introduce artefactual alterations in the amounts of Figlu and urocanic acid (Luhby and Cooperman, 1964).

We have recently reported the use of ascending chromatography on thin layers of cellulose as a relatively simple, sensitive, and rapid method for simultaneous separation and semi-quantitative estimation of Figlu and urocanic acid (Mohamed and Roberts, 1964; Roberts and Mohamed, 1965). The

method has now been used to estimate the urinary excretion of these two metabolites on histidine loading in a representative group of cases and the results have been examined to assess the claim of Bennett and Chanarin in respect of urocanic acid.

PATIENTS, MATERIALS, AND METHODS

PATIENTS The 64 subjects studied are listed in Table I.

The 15 control subjects were eight healthy adult members of the staff, and seven patients, who had no evidence of any gastrointestinal or haematological abnormality or conditions likely to produce folate deficiency.

All five patients with the malabsorption syndrome were anaemic with a 'dimorphic' peripheral blood picture, but only three showed megaloblastic erythropoiesis. Three patients had a histamine-fast achlorhydria. In two of these, and in one patient with free acid, a jejunal biopsy was performed, and this showed partial or subtotal villous atrophy. All five patients showed flat glucose tolerance curves, subnormal excretion of D-xylose, and a flocculation pattern on x-ray examination of the small intestine, but only four had frank steatorrhoea. The fat excretion in the remaining patient averaged 4.8 g./day. Of the four patients in whom it was tested, vitamin B₁₂ absorption was subnormal in two but was not in the range characteristic of pernicious anaemia. In only one of these could be corrected by the administration of hog intrinsic factor. A complete haematological response to folic acid was obtained in four of the five patients after an initial sub-optimal response to vitamin B₁₂. The remaining patient, showing a mild dimorphic anaemia with normoblastic erythropoiesis, responded to a gluten-free diet alone.

All six patients with pernicious anaemia were previously untreated cases who had megaloblastic anaemia, histamine-fast achlorhydria, and a defect of vitamin B₁₂ absorption which could be corrected by giving hog intrinsic factor. A classical response to vitamin B₁₂ therapy occurred in each case.

The patient with Crohn's disease was a young woman of 24 with moderate hypochromic, normoblastic anaemia, a flat glucose tolerance curve, and malabsorption of D-xylose and fat. She had radiological evidence of regional ileitis involving the terminal ileum. An 18-inch portion of the diseased bowel was subsequently resected.

The 17 patients with partial gastrectomy all had had a Polya repair five or more years earlier and none was anaemic at the time of the test.

Cirrhosis of the liver in both patients was of the cryptogenic variety, and both had liver cell failure, with moderate normochromic anaemia, leucopenia, and thrombocytopenia. The patient with cardiac failure was a woman of 84 with valvular heart disease and moderate iron-deficiency anaemia.

Of the nine patients with thyrotoxicosis, two had associated diseases; one, a boy of 15, had an iron-deficiency anaemia with splenomegaly, and he responded well after treatment with iron; and the other was a man of 54 who had undergone a mitral valvotomy 18 months earlier and had subsequently remained free of congestive failure, auricular fibrillation being controlled with digitalis.

TABLE I
SEVENTY-FOUR HISTIDINE LOAD TESTS IN 64 SUBJECTS

Case No.	Condition	No. of Cases	Histidine Metabolites (mg./8 hr.) ¹		
			Figlu	Urocanic Acid	Total
1 to 15	'Control' subjects	15 (24 tests)	0-30	0-20	0-35
16	Malabsorption syndrome	5	140	50	190
17			350	15	365
18			350	90	440
19			140	0	140
20			120	50	170
21 to 24	Pernicious anaemia	6 {	Normal 4 0-15	5-20	5-25
25			Abnormal 2 140	70	210
26			140	0	140
27 to 40	Partial gastrectomy	17 {	Normal 14 0-30	0-10	0-30
41			Abnormal 3 70	20	90
42			0	50	50
43			0	70	70
44	Crohn's disease	1	0	60	60
45	Cirrhosis of liver	2	70	100	170
46	Cirrhosis of liver		50	150	190
47	Congestive failure: valvular heart disease	1	0	90	90
48 to 50	Thyrotoxicosis	9 {	Normal 3 0-15	0-15	0-30
51			Abnormal 6 0	45	45
52			80	160	240
53			10	80	90
54			50	0	50
55			0	80	80
56			90	10	100
57	Carcinoma of bronchus	3	80	10	90
58	Carcinoma of bronchus		30	140	170
59	Carcinoma of bronchus		0	0	0
60	Carcinoma of stomach	1	35	5	40
61	Acute monocytic leukaemia	1	160 (180) [*]	25 (30) [*]	185 (210) [*]
62	Malignant myelosclerosis	1	50	80	130
63	Myelofibrosis: pernicious anaemia	1	120	60	180
64	Erythraemic myelosis	1	25	950	975

¹Excretions of Figlu (formiminoglutamic acid) and urocanic acid in the different disease groups are expressed as the observed range when the results were normal; individual values are shown where the combined excretion of the two metabolites was in excess of normal.

^{*}Results of repeat tests.

The last group of patients had malignant disease. There were three cases of bronchial carcinoma, and a case each of gastric carcinoma, acute monocytic leukaemia, malignant myelosclerosis, erythraemic myelosis, and myelofibrosis. The last patient, whom we have reported elsewhere (Roberts and Mohamed, 1965), also showed a megaloblastic anaemia with defect of vitamin B₁₂ absorption of the type found in pernicious anaemia.

TEST URINES These were collected over an eight-hour period after a loading dose of 15 g. of L-histidine monohydrochloride. Aliquots of the measured total volume were stored at -20°C. until the time of the assay.

ESTIMATION OF FIGLU AND UROCANIC ACID This was carried out by the semi-quantitative method on thin-layer chromatograms, as described previously (Mohamed and Roberts, 1964; Roberts and Mohamed, 1965).

RESULTS

The eight-hour excretions of Figlu and urocanic acid

in 24 tests in the 15 control subjects are shown individually in Figure 3. The mean combined excretion of the two metabolites was 13.8 mg. and the mean + 2.32 standard deviation level, *i.e.*, the level which would be expected to be exceeded by a normal subject once in 100 tests, was 38.6 mg., or approximately 40 mg.

Using the 40 mg. level for the combined excretion as the discriminant, it can be seen in Table I that excretions in excess of this were found in all five patients with the malabsorption syndrome, in two of the six patients with pernicious anaemia, in the one patient with Crohn's disease, and in three of the 17 patients who had had a Polya gastrectomy. Both the patients with cirrhosis of the liver exceeded this figure, as did the one patient with cardiac failure, six of the nine thyrotoxic patients, and six of the eight patients with the various malignant diseases.

Table II shows the contribution of urocanic acid

Fig. 3. Individual results of 24 histidine load tests in 15 control subjects. UA = urocanic acid; Figlu = formiminoglutamic acid. The mean and mean $\pm$ 2.32 standard deviation (S.D.) range of the combined excretion of Figlu and urocanic acid are shown.

to the total histidine metabolites observed in the individual diagnostic groups of cases. Of the 74 tests in 64 subjects, urocanic acid was present in all but 27% of the tests. In 29.8% of the tests urocanic acid was present in amounts in excess of Figlu. In fact, urocanic acid was the only metabolite present in 12 of the tests, and in six of these tests the amounts were considerably in excess of the normal excretion.

TABLE II

CONTRIBUTION OF UROCANIC ACID TO HISTIDINE METABOLITES IN URINE

Diagnosis	No. of Tests	Urocanic Acid as Percentage of Total Histidine Metabolites ¹				
		0	1-25	26-50	51-75	76-100
Normal controls	(15) ² 24	8	4	5	1	6
Steatorrhoea	(5) 5	1	1	3	0	0
Pernicious anaemia	(6) 6	1	0	3	0	2
Crohn's disease	(1) 1	0	0	0	0	1
Partial gastrectomy	(17) 17	6	6	2	0	3
Cirrhosis of liver	(2) 2	0	0	0	2	0
Cardiac failure	(1) 1	0	0	0	0	1
Thyrotoxicosis	(9) 9	3	2	1	1	2
Malignancies (various)	(8) 8	1	5	0	1	2
Total	(64) 74	20	18	14	5	17
		(27.0%)	(43.2%)		(29.8%)	

¹Numbers shown refer to actual numbers of tests in each group for the different ranges of excretion of urocanic acid expressed as a percentage of total histidine metabolites.

²Figures in brackets refer to number of cases in each group.

The statistical ranges of excretion of Figlu and urocanic acid in the 24 tests in the control subjects are shown in Table III. The upper limits of excretion

TABLE III

STATISTICAL RANGES OF EXCRETION OF FIGLU, UROCANIC ACID, AND THE COMBINED EXCRETION OF THE TWO METABOLITES IN CONTROLS

Histidine Metabolite (mg./8 hr.)	Mean ($\bar{x}$)	Standard Deviation ($\bar{\sigma}$)	$\bar{x} \pm 2.32\sigma$
Figlu	9.0	8.6	0-28.95 (approx. 30 mg.)
Urocanic acid	4.8	4.99	0-16.38 (approx. 15 mg.)
Figlu + urocanic acid	13.8	10.7	0-38.6 (approx. 40 mg.)

TABLE IV

DISCRIMINANT VALUE OF FIGLU, UROCANIC ACID, AND THE COMBINED EXCRETION OF THE TWO METABOLITES

Diagnosis	No. of Tests	No. of Abnormal Tests for Figlu (>30 mg.)	No. of Abnormal Tests for Urocanic Acid (>15 mg.)	No. of Abnormal Tests for Figlu + Urocanic Acid (>40 mg.)
Normal controls	(15) ¹ 24	—	1	—
Steatorrhoea	(5) 5	5	3	5
Pernicious anaemia	(6) 6	2	2	2
Crohn's disease	(1) 1	0	1	1
Partial gastrectomy	(17) 17	1	3	3
Cirrhosis of liver	(2) 2	2	2	2
Cardiac failure	(1) 1	0	1	1
Thyrotoxicosis	(9) 9	3	4	6
Malignancies (various)	(8) 8	6	6	7
Total	(64) 74	19	23	27
		(70.3%)	(85.2%)	(100%)

¹Figures in brackets refer to the number of cases studied in each group. Upper limits of excretion of Figlu, urocanic acid, and Figlu + urocanic acid in the normal are statistically derived as shown in Table III.

(mean $\pm$ 2.32 standard deviation) were approximately 30 mg. for Figlu, 15 mg. for urocanic acid and 40 mg. for the combined excretion of the two metabolites. As can be seen in Table IV, of the 24 tests with abnormal combined excretion of Figlu and urocanic acid, only 19 (70.3%) can be identified by the estimation of Figlu alone. Simultaneous measurement of urocanic acid, allowed an additional eight cases to be identified as showing an abnormality of histidine metabolism.

DISCUSSION

Subclinical deficiency of folate has been shown to be very common in a variety of clinical conditions, and Luhby and Cooperman (1964) estimate that folate deficiency may be 'among the most prevalent, if not the most common, of the vitamin deficiencies of present-day man'. Serum folate levels were not estimated in our patients, but the results of the histidine load tests in the malabsorption syndrome and pernicious anaemia are in conformity with

general experience with the Figlu test (Chanarin, 1960; Carter, Heller, Schaffner, and Korn, 1961; Herbert and Zalusky, 1962; Knowles and Prankerd, 1962; Chanarin, Bennett, and Berry, 1962; Luhby and Cooperman, 1964; Kershaw and Girdwood, 1964). The abnormal results in three of the 17 patients who had a Polya gastrectomy are comparable with the 10.4% incidence of folate deficiency, as assessed by bio-assay of serum folate activity (Deller, Ibbotson, and Crompton, 1964). These cases will be described in detail elsewhere.

Interference with folate metabolism, or a relative deficiency because of increased utilization, as assessed by increased urinary excretion of Figlu, subnormal urinary excretion of an oral dose of folic acid, rapid plasma clearance of an intravenous dose of folic acid, decreased liver content of folate, or even the presence of megaloblastic anaemia, have been described in malignant disease (Ehrlich and Lazarus, 1900; Swendseid, Swanson, Meyers, and Bethell, 1952; Girdwood, 1953; 1959; Broquist, 1956; Chanarin, Mollin, and Anderson, 1958; Chanarin *et al.*, 1962; Romine, 1960; Chanarin, 1963; Villamil and McCracken, 1963; Karlin, 1963; Carey, Brena, and Krant, 1964; Kershaw and Girdwood, 1964; Dymock, 1964). Increased levels of Figlu and/or urocanic acid have been noted in cirrhosis of the liver (Luhby *et al.*, 1959a; Carter, Schaffner, and Heller, 1960; Carter *et al.*, 1961; Merritt, Rucknagel Silverman, and Gardiner, 1962; Knowles, Shaldon, and Fleming, 1963; Villamil and McCracken, 1963; Kershaw and Girdwood, 1964) and recently excessive amounts of Figlu have also been reported in patients in cardiac failure (Daly and Rose, 1964).

The abnormal results in six of the nine thyrotoxic patients, without significant complicating diseases, are of interest and these cases have been reported (Mohamed and Roberts, 1965). Theoretically, there are several ways in which thyrotoxicosis may interfere with folate metabolism: an increased requirement for folate consequent upon the general enhancement of metabolism or the known increase in red cell mass (Muldowney, Crooks, and Wayne, 1957) could account for it. The possibility of liver cell dysfunction in thyrotoxicosis (Cameron and Karunaratne, 1935; Maddock, Collier, and Pedersen, 1936; Shaffer, 1940; McIver and Winter, 1943; Piper and Poulsen, 1947; Myers, Brannon, and Holland, 1950; Movitt, Gerstl, and Davis, 1953) also merits consideration. It is of interest to note that low liver folate levels have been noted in rats rendered 'thyrotoxic' by feeding with iodinated casein (Noronha and Sreenivasan, 1959). Deficiency of Figlu transferase (Luhby and Cooperman, 1964) has been postulated in cirrhosis of the liver and in rheumatoid arthritis and this may also apply to

thyrotoxicosis in man. Further studies to evaluate the possible role of some of these factors in thyrotoxicosis are in progress.

We think it reasonable to assume that the excessive combined excretion of Figlu and urocanic acid noted in 27 of the 64 patients in this series reflects either an absolute or relative deficiency of folate, or at any rate some abnormality of folate metabolism. Of these, only 19 patients were identified by measurement of urinary Figlu alone. Furthermore, urocanic acid was present in 73% of the 74 tests and in 29.8% of the tests the amounts were in considerable excess of Figlu. Six of the control subjects excreted only urocanic acid, as did six of the 27 patients with abnormal results.

These results are in strong agreement with those reported by Bennett and Chanarin and reinforce their suggestion that both Figlu and urocanic acid should be estimated in histidine load tests as an index of folate deficiency.

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