Clinical and Experimental Evaluation of Urinary Histidine Derivatives as an Index of Folic Acid Metabolism

I. The Observation in Hematological Disorders

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A modified enzyme method for the simultaneous estimation of urinary formiminoglutamic acid (FIGLU) and urocanic acid after a loading with 15 g of L-histidine has been applied to 10 normal subjects and 40 patients with various blood disorders. The 24-hour excretion of the two derivatives in normal subjects was within 30 mg for each of them and within 60 mg for the combined two derivatives. FIGLU in excess was found in 45 % of 40 patients, and urocanic acid in excess, in 40 % of 25 patients. All of six patients whose hematopoieses were megaloblastic excreted excessive FIGLU or the combined two derivatives. Out of 12 cases in whom the total amount of histidine derivatives exceeded the normal range of 60 mg, 11 cases were in excess of FIGLU, and 10 cases, of urocanic acid excretion; 3 cases excreted only FIGLU in excess, and 3 cases, only urocanic acid in excess. Out of these 12 cases, six cases excreted both FIGLU and urocanic acid in excess, and urocanic acid was the predominant constituent in one patient. These results suggested a wide-spread existence of folic acid deficiency in blood disorders, and simultaneously, supported the value of estimating both the histidine derivatives for detecting folate deficiency in humans.

The degradation of histidine to glutamic acid proceeds via two relatively stable intermediate compounds, urocanic acid and formiminoglutamic acid (FIGLU). At the same time, tetrahydrofolic acid (THF) is required for the conversion of FIGLU to glutamic acid, and when there is any interference with normally functioning folic acid coenzyme, these histidine derivatives are accumulated and excreted in the urine (1). More interest was enhanced by the fact that the amount metabolized to glutamic acid could be augmented by a suitable histidine loading (21,31). Therefore, the evaluation of histidine derivatives can apparently be employed as a sensitive,

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FIG. 1 *The Schematic Degradation of Histidine and Its Relation to the Assay Methods.*

reliable and rapid test for biochemical folic acid deficiency in man. For the estimation of these compounds, four kinds of methods, microbiological, enzymic, chromatographic, and chemical assays have been reported. The interrelationship between the metabolic fate of histidine and the assay methods is summarized in Fig. 1.

FIGLU was first detected by microbiological assay in human subjects treated with folic acid antagonists (6), and in patients with macrocytic anemia of pregnancy (20). In earlier studies, most interest was focussed on FIGLU, and further observations confirmed these findings and extended them to a variety of other clinical folic acid deficiency states.

However, urocanic acid, the precursor of FIGLU, which had been identified in the urine of small mammals and of man (28, 29), received a great deal of attention because of its appearance on histidine loading in patients with folic acid deficiency (3). Bennett and Chanarin have reported that urocanic acid comprised more than 80 % of histidine derivatives excreted in the urine in megaloblastic anemia (3), and the subsequent studies revealed increased excretion of urocanic acid in various clinical disorders (10, 34).

It has been also demonstrated that urocanic acid appeared in the urine approximately in an equal amount when an adequate dose of histidine was loaded, and that the amount of urocanic acid exceeded that of FIGLU in a considerable number of patients, in several cases urocanic acid being the only compound excreted (9).

The purpose of this study was, first, to reexamine the spectrophotometric method by Chanarin and Bennett (10) for the estimation of both urocanic acid and FIGLU, and secondly, to observe the urinary excretion of these histidine derivatives in patients with various hematological disorders.

Furthermore, the effects of folic acid and vitamin B₁₂ on urinary excretion of FIGLU and urocanic acid were observed in some cases.

METHOD AND MATERIALS

The methods for the estimation of urocanic acid and FIGLU were essentially the same as those described by Chanarin and Bennett (10).

The assay consists of the following steps. *Step 1 (preincubation)*: equal amounts of liver extract and substrate solution, containing folic acid, NADP, minerals and phosphate buffer, were mixed, and 3 ml of the solution was incubated for 1-2 hours at 37°. This procedure provided THF for the next reaction. *Step 2 (incubation)*: the heated or unheated urine to be tested was added in the volume of 0.05 to 0.2 ml to the mixture of step 1. Distilled water was added to make the total volume 4.0 ml. The next incubation for 2 hours at 37° was immediately performed. The end product of this incubation is N^{10} -CHO-THF. *Step 3*: at the end of step 2, 1.5 ml of 3 *N* HCl was added and the mixture was filtered. The filtrate contains N^5 , N^{10} -methenyl THF. *Step 4*: N^5 , N^{10} -methenyl THF was read in a Beckman Spectrophotometer at 350 m μ .

1. Materials

Enzyme preparation—A crude liver enzyme preparation by Futterman was used (12). Frozen chicken liver was sliced, added to 1.3 volume of 0.1 *M* potassium phosphate buffer, pH 6.0, and homogenized for about 10 seconds. The homogenate was centrifuged at 33,000 $\times g$ for 10 minutes and the supernatant was dialyzed against water overnight. The pH was adjusted to 6.0 and the insoluble residue was removed by centrifugation. All operations were carried out at 0 to 4°. The extract was stored at -20°.

Substrate solution—It contained folic acid (55 μ moles), NADP (12 μ moles), KH_2PO_4 (20 μ moles), citric acid (2 μ moles), and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (2 μ moles) in 100 ml of distilled water at pH 6.0, and was stored at -20°. In addition, to stabilize THF during these procedures, 10⁻² *M* 2-mercaptoethanol was added.

Urine samples—Urine was collected for 24 hours following a single oral dose of 15 g of L-histidine hydrochloride. The urine containers had been added with 5 ml of 3 *N* HCl to prevent the destruction of FIGLU.

Hydrolysis of urine—A part of urine samples collected was first heated at 100° for 10 minutes at pH 11.0-12.0. By this procedure, more than 98 % of FIGLU in the urine could be destroyed.

FIGLU and urocanic acid for standard solutions—Barium salt of FIGLU and urocanic acid were purchased commercially from Sigma Co.

2. Procedures

The details of the method used in this study were almost the same as described by Chanarin and Bennett in 1962 (10); the modified method is shown briefly in Fig. 2.

Because the enzymatic activity of crude liver extract was a little changeable according to several conditions in the course of preparation procedures, the time of preincubation had to be varied from 1 to 2 hours to obtain the maximum and most stable reaction in the following procedures.

The urine volume had to be varied from 0.05 to 0.4 ml so that the amounts of FIGLU and urocanic acid in the urine samples should not exceed 0.3 μM .

FIG. 2 *The Modified Method for the Measurement of Urinary FIGLU and Urocanic Acid*

was used in the assay of urocanic acid. After preincubation of the enzyme and substrate solution, 1.5 ml of 3 N HCl was added to the urine sample in a blank, followed by the incubation for 2 hours. By this procedure, the blank tube contained the same amount of the material and the error in the measurement of urocanic acid was corrected.

TABLE 1
Scheme for Estimation of FIGLU and Urocanic Acid

	(A) Test for FIGLU	(B) Test for urocanic acid	(C) Enzyme blank	(D) Blank for urocanic acid	(E) Standard for FIGLU	(F) Standard for urocanic acid
Substrate solution	+	+	+	+	+	+
Liver enzyme	+	+	+	+	+	+
Unhydrolyzed urine	+	—	—	—	—	—
Hydrolyzed urine	—	+	—	—	—	—
3 N HCl added before incubation	—	—	—	+	—	—
FIGLU	—	—	—	—	+	—
Urocanic acid	—	—	—	—	—	+
0.1 M potassium phosphate buffer	Total volume to 3 ml with water					

(A), (B), (E), and (F) are read against the blank (C). (A-B) is the optical density of FIGLU and the amount of it is calculated from the reading of (E).

(B) is read against (D); (B-D) correspond to the optical density of urocanic acid. Standard of urocanic acid is (F).

3. Patients Studied

Patients with a variety of blood disorders were studied and the urinary excretion of histidine derivatives was estimated. Forty cases were examined, including two cases of pernicious anemia, two of blind loop syndrome, five of aplastic anemia,

four of anemia due to iron deficiency, two of hemolytic anemia, six of postgastrectomy, 12 of leukemia, two of SLE, four of Hodgkin's disease, and one of myeloma. In 31 of them, bone marrow aspiration was performed and the bone marrow picture was examined.

FIG. 3 *Optical Density Obtained when Increasing Amounts of FIGLU and Urocanic Acid Were Added to Water.*
—, FIGLU; ···, urocanic acid.

RESULTS

1. Recovery of Histidine Derivatives Added to Urine or Water

The standard curves of FIGLU and urocanic acid at 350 $\mu\mu$, found after addition of increasing amounts of the derivatives to the enzyme system, is shown in Fig. 3. The extinction of urocanic acid was observed to run parallel to, and a little less than that of FIGLU. The standard curve using barium salt of FIGLU and urocanic acid as reference was made in every assay.

Recovery of FIGLU Added to Water—Table 2 shows the recovery test of FIGLU in the volume of 0.1 μ mole per ml without urine, for the purpose of comparing the effect of 2-mercaptoethanol on the assay. In the

TABLE 2
Effect of 2-Mercaptoethanol on the Recovery of FIGLU

No. of tube	FIGLU (0.1 μM)	2-Mercaptoethanol ($10^{-2} M$)	FIGLU measured μM	Recovery per cent
1	+	+	0.967	96.7
2	+	+	1.025	102.5
3	+	+	1.014	101.4
4	+	+	0.989	98.9
5	+	+	0.993	99.3
6	+	+	1.018	101.8
7	+	+	1.014	101.4
8	+	+	1.036	103.6
9	+	+	1.007	100.7
10	+	+	0.986	98.6
Mean $\pm$ standard deviation :			1.000 $\pm$ 0.058	
1	+	—	1.026	102.6
2	+	—	1.021	102.1
3	+	—	1.034	103.4
4	+	—	0.945	94.5
5	+	—	0.992	99.2
6	+	—	0.971	97.1
7	+	—	1.018	101.8
8	+	—	1.003	100.3
Mean $\pm$ standard deviation :			1.000 $\pm$ 0.083	

tubes without 2-mercaptoethanol recovery ranged from 94.5 % to 103.4 % and in the tubes with it, from 96.7 % to 103.6 % with improvement of standard deviation from $\pm 8.3\%$ in the former to $\pm 5.8\%$ in the latter.

Recovery of urocanic acid added to urine—Table 3 shows the recovery of urocanic acid added to the urine of randomly selected ten normal subjects. The samples added with 0.05 or 0.2 μM urocanic acid to the urine were measured with the urine blank and HCl blank, and the recoveries by the two methods were compared. The urine blank means the extinction of the urine against water. The urine samples containing urocanic acid were read against the enzyme blank, and from this extinction the value of urine blank was subtracted. The difference is the extinction of urocanic acid. As for HCl blank, it was fully described before. The extinction of urocanic acid was measured from the reading of the same urine samples against HCl blank.

Recoveries of urocanic acid using 0.2 and 0.05 μM was 92.5 and 74.2 % in the case of urine blank, and 102.0 and 115.6 % in the case of HCl blank, respectively.

TABLE 3
Recovery of Urocanic Acid by Use HCl Blank and Comparison with That of Urine Blank

No. of	Optical density of tested urine against water (urine blank)	Optical density of HCl blank against enzyme blank	Percentage recovery of urocanic acid against urine blank		Percentage recovery of urocanic acid against HCl blank	
			0.2 μM	0.05 μM	0.2 μM	0.05 μM
1	0.061	0.008	92.7	73.8	101.6	109.2
2	0.196	0.091	88.4	49.2	105.9	113.0
3	0.062	0.017	91.6	70.0	102.0	113.0
4	0.071	0.029	96.9	94.6	103.0	111.5
5	0.080	0.270	96.5	83.1	104.3	118.5
6	0.052	0.008	96.1	80.8	104.0	116.2
7	0.231	0.147	82.7	47.8	102.7	119.2
8	0.045	0.002	92.4	82.3	101.9	117.0
9	0.056	0.002	91.4	68.5	101.0	111.5
10	0.237	0.170	99.6	92.3	104.0	123.1
Average of 10 tubes:			92.5	74.2	102.0	115.6

HCl blank: After usual procedure of preincubation, the urine was added to the enzyme system with 1.5 ml of 3 *N* HCl, and the usual incubation was performed.

2. Urinary Excretion of Histidine Derivatives in Patients with Blood Disorders

Firstly, the 24-hour excretion of FIGLU and urocanic acid was determined in ten control subjects. The mean excretion was 9.9 mg of FIGLU and 10.2 mg of urocanic acid with the standard deviation of 5.57 and 5.67 mg respectively. The normal upper limit of the two derivatives was 26.6 mg of FIGLU and 27.1 mg of urocanic acid or approximately 30 mg of each.

The estimation of FIGLU was made in 40 patients, of whom 25 were included for determination of urinary urocanic acid excretion. As indicated in Table 4, abnormal histidine derivative excretion appeared frequently in various blood disorders, especially in pernicious anemia, postgastrectomy, CML, and Hodgkin's disease. Of 40 patients examined, 18 (45 %) excessive FIGLU, and of 25 patients, ten (40 %)

TABLE 4
Hematological Findings and Histidine Derivatives in Patients with Blood Disorders

No. of case	Diagnosis	Hematological findings					Histidine derivatives	
		RBC	WBC	Hb	Ht	Megalo blast	FIGLU	Urocanic acid
		$\times 10^6$	$\times 10^3$	g	per cent		mg/day	
1	Pernicious anemia	1.18	3.4	5.8	21	+	116	16
2	"	1.65	2.2	5.9	19	+	451	28
3	Blind loop syndrome	2.25	5.8	5.8	28	+	25	139
4	"	2.60	6.5	6.5	32	+	49	
5	Aplastic anemia	2.63	3.9	7.6	22	—	13	
6	"	3.54	3.6	13.1	38	—	6	
7	"	2.69	3.7	8.7	25	—	29	6
8	"	3.03	4.9	11.4	33	—	4	
9	"	1.96	2.1	6.7	24	—	14	30
10	Iron deficiency	3.34	5.3	6.6	23		14	8
11	"	3.06	7.1	8.8	30		4	10
12	"	2.60	4.7	5.3	19	—	81	
13	"	1.70	5.0	6.3	24		37	
14	Hemolytic anemia	2.46	4.5	10.1	28	—	0	
15	"	3.39	7.5	12.5	38	+	84	59
16	Postgastrectomy	2.75	4.2	8.7	27	—	7	58
17	"	2.95	5.4	11.4	35	+	57	54
18	"	2.31	3.5	7.7	31		64	5
19	"	2.57	5.8	8.9	29		24	19
20	"	2.98	9.1	9.3	38		384	
21	"	3.15	4.8	12.5	37		175	
22	A M L	4.03	7.9	13.6	40	—	9	13
23	"	4.86	3.3	15.1	45	—	18	15
24	"	3.88	64.8	11.5	39	—	29	19
25	"	3.29	5.5	10.4	29	—	12	21
26	"	2.90	11.8	8.6	30	—	25	
27	C M L	2.32	28.4	7.0	25	—	65	37
28	"	4.25	11.6	11.4	31	—	84	12
29	"	3.14	19.1	8.4	25	—	24	121
30	"	3.00	55.6	17.2	32	—	0	
31	"	5.93	64.4	15.8	45	—	243	
32	"	2.39	2.3	6.8	21	—	50	5
33	"	3.63	16.9	13.0	40	—	14	
34	S L E	3.76	6.0	10.5	25	—	24	
35	"	3.84	8.4	11.9	42		25	34
36	Hodgkin's disease	3.36	2.2	8.2	28	—	252	
37	"	3.18	4.5	11.2	36	—	117	323
38	"	3.12	5.4	8.8	23	—	162	49
39	"	3.06	6.2	7.9	22		445	61
40	Myeloma	2.97	9.4	9.6	35	—	7	13

was found to excrete an abnormal amount of urocanic acid. The excretion of the two or FIGLU alone in excess was found in all the patients with pernicious anemia and Hodgkin's disease, in four of six patients with postgastrectomy, in four of seven patients with CML, in two of four patients with anemia due to iron deficiency, and in one of two patients with hemolytic anemia. All the patients with aplastic anemia, AML, SLE, and myeloma excreted a normal amount of the derivatives.

Regarding the hematological findings of 40 patients in Table 4, no firm, mutual relationship was found among the amounts of histidine derivatives and red and white blood cell count, hematocrit, and hemoglobin concentration. Likewise, no proportional correlation was observed between the amounts of the derivatives and the degree of hematological abnormality in individual blood diseases.

However, all of six patients, whose bone marrow were megaloblastic, excreted excessive FIGLU or both derivatives. These six patients included each two cases with pernicious anemia and blind loop syndrome and each one case with postgastrectomy and hemolytic anemia.

Table 5 shows the urinary excretion of histidine derivatives and the frequency of the abnormal excretion in blood disorders for demonstrating the value of urocanic acid test. Out of 12 patients, excreting the derivatives beyond the normal upper limit of 60 mg, ten cases showed abnormal urocanic acid excretion, and 11 cases, abnormal FIGLU excretion (both over 30 mg). The concurrent appearance of an abnormal amount of FIGLU as well as urocanic acid was noticed only in six cases, and in one case with Hodgkin's disease urocanic acid exceeded the amount of FIGLU.

On the other hand, either FIGLU or urocanic acid was the only derivative exceeding the upper limit of 60 mg in the remaining six cases; the former was observed in three cases and the latter in three cases. Similarly, these three cases excreting the excessive urocanic acid only were found in the patients with blind loop syndrome, postgastrectomy and hemolytic anemia, respectively.

TABLE 5
Urinary Excretion of Histidine Derivatives in Blood Disorders

Diagnosis	No. of cases	Histidine derivatives in urine (no. of abnormal cases)		
		FIGLU (A)	Urocanic acid (B)	A + B
Normal controls	10	—	—	—
Pernicious anemia	2	2	0	2
Post-gastrectomy	4	2	2	3
Blind loop syndrome	1	0	1	1
Aplastic anemia	2	0	0	0
Leukemia				
AML	4	0	0	0
CML	4	3	2	2
Hodgkin's disease	3	3	3	3
Myeloma	1	0	0	0
SLE	1	0	1	0
Iron deficiency	2	0	0	0
Hemolytic anemia	1	1	1	1
Total	25	11	10	12
Per cent		44	40	48

3. Effect of Administration of Folic Acid and Vitamin B₁₂ on Urinary Excretion of Histidine Derivatives and on Clinical Findings

Two cases were chosen for observing the effect of folic acid and vitamin B₁₂ on the urinary histidine derivatives, and comparing the effect with haematological response.

Case I (Fig. 4) — a male aged 32, gave a history of having had hyperthyroidism about nine years before admission. His sternal bone marrow aspirate showed a typical megaloblastic change (megaloblast; 55.6 %) on admission. The examination of blood disclosed 38 % hemoglobin, 1.8×10^6 red blood cell count and 2,100 white blood cell count with hypersegmentation. The urinary excretion of histidine derivatives was 479 mg/day, and FIGLU was the predominant constituent (451 mg) before treatment.

FIG. 4 Return of Urinary Histidine Derivatives to within Normal Range after a Dose of Folic Acid and of Vitamin B₁₂ in a Patient with Pernicious Anemia.

Correlation between the response of the derivatives and hematological findings was summarized.

■, amount of FIGLU; □, amount of urocanic acid.

Initially, he was treated with a single, parenteral dose of 60 mg folic acid. Immediately after the dose, a decline of urinary histidine derivatives occurred, but still a large amount was excreted. The hematological response to the dose of folic acid was a slight increase of reticulocyte count, and megaloblast in bone marrow remained unchanged (megaloblast; 63.2 %).

On the contrary, the dose of 10 mg coenzyme B₁₂² and following 1,000 μg of CN-B₁₂³ resulted in a marked decrease of histidine derivatives, which returned to a normal range, with a marked increase of reticulocyte count. Megaloblasts in bone marrow decreased to 32.4 % after a dose of coenzyme B₁₂, and disappeared after a dose of CN-B₁₂. On the 30th day after the initiation of the vitamin B₁₂ administration, red blood cell count rose to 4.2×10^6 and hemoglobin to 75 %. Histidine derivatives never exceeded the normal limit in the repeated estimations.

Case 2 (Fig. 5) — The patient, a male aged 60, had a total gastrectomy for stomach ulcer 15 years before admission. The urinary excretion of histidine deriva-

² 5,6-Dimethylbenzimidazolylcobamide coenzyme

³ Cyanocobalamin

FIG. 5 Return of Urinary Histidine Derivatives Excretion after a Relatively Small Dose of Folic Acid in a Patient with Pernicious Anemia.

■, amount of FIGLU, □, amount of urocanic acid.

tives which was 132 mg/day on admission fell to a normal value of 43 mg, following a single, small dose (0.76 mg) of folic acid. However, the comparison of bone marrow aspiration examined before and 24 hours after the dosage of folic acid revealed no effective influence of folic acid on megaloblast. The examination of blood also disclosed the unchanged value of red blood cell count (from 1.32 to 1.46×10^6) and hemoglobin concentration (from 35 to 36 %) except for a moderate increase of reticulocyte count. On the contrary, the disappearance of megaloblast was confirmed 24 hours after a subsequent dose of $5 \mu\text{g}$ of CN- B_{12} . An immediate reticulocytosis followed by an increase of red blood cell count to 2.75×10^6 was found, and histidine derivatives still remained within normal.

After these procedures, ^{60}Co -CN- B_{12} absorption was measured by the method of Schilling with or without the use of intrinsic factor. He excreted 1.42 % of ^{60}Co -CN- B_{12} when the dose was given alone, but showed an enhanced excretion of 7.5 % up to low normal level when it was given with intrinsic factor. In parallel with this, an increase of red blood cell count up to 3.85×10^6 and a moderate reticulocytosis was observed.

From the results of the serial, hematological observations, it was apparent that these two cases were suffering from vitamin B_{12} deficiency. However, the folic acid administration succeeded to abolish the increased urinary excretion of histidine derivatives in both of the cases, though their megaloblast in bone marrow failed to respond to folic acid.

On the other hand, CN- B_{12} was as effective as folic acid in the case 2, and successfully decreased the megaloblast in both of the cases.

DISCUSSION

The four assay methods, microbiological (7, 14, 26), enzymic (26, 31, 32), chromatographic (5, 30), and chemical (32) that have been used for estimating urinary FIGLU or urocanic acid, differ considerably in sensitivity and specificity. The predominant methods currently employed are the enzymic assay which has the sufficient sensitivity to quantitate urinary FIGLU or urocanic acid over the wide range of clinically diagnostic concentrations.

Three methods are available for enzymic assay, *i.e.*, the T-C enzyme assay described by Tabor and Wyngarden (32), the assay described by Silverman (27), and Chanarin and Bennett's modification of the latter (10). Of these, the T-C enzyme assay is the most sensitive and simultaneously, biochemically most specific method for the estimation of FIGLU, but has the disadvantage of requiring the highly unstable THF, and the unsuitability for measurement of urocanic acid.

Although the correlation between folate deficiency and increased urinary urocanic acid excretion was observed to be less uniform than its correlation with increased urinary FIGLU excretion because of evidence of several factors such as urocanase activity in liver (2), or backup phenomenon of FIGLU, the importance of concomitant estimation of the excretion of both FIGLU and urocanic acid led to the adoption of the method of Chanarin and Bennett in this study.

However, it has been pointed out that the method has some factors of technical error which have been inadequately investigated for the measurement of urocanic acid. For example, the crude liver extract for the enzyme preparation in this assay contains a considerable histidase capable of converting histidine in the test urine to urocanic acid (22). Therefore, some of the elevated urinary urocanic acid levels in the assay may result from an artificial source. In addition, it was found in the preceding report (31) that the enzyme preparation had the ability of removing some material from the test urine absorbing in the region of 350 m μ . Although the report indicated that the error was equivalent to 2-5 mg of FIGLU, an amount well within the experimental error, the problem was reexamined by the author and found that the error could not be negligible. For correction, the HCl blank was introduced which resulted in a considerable improvement.

The recovery of urocanic acid using HCl blank was relatively high in contrast with the low values using urine blank and the result of this study showed the use of HCl blank to improve the correctness of the measurement of urocanic acid. Further, application of 2-mercaptoethanol was shown to be beneficial for prevention of the destruction of THF, whose stabilization was the most important factor for a quantitative analysis. However, the problem about the existence of histidase in the liver extract has not been solved, and further evaluation is needed.

As a biochemical index of folate deficiency, the estimation of urinary FIGLU or histidine derivatives has become firmly established, and is a more sensitive and reliable index in folate deficiency states than any of other parameters. Simultaneously, it can be found in reports from all parts of the world that the incidence of folate deficiency is much higher than previously suspected, occurring in a variety of clinical conditions for which evidence was formerly absent or incomplete. In most of these conditions, the morphological changes such as megaloblastosis are

often minimal or equivocal and folate deficiency is demonstrable only by increased urinary histidine derivatives after histidine loading, presumably reflecting the great sensitivity of this examination. The present study demonstrated that the bone marrow of six patients was megaloblastic, and the urinary excretion of histidine derivatives was abnormal. However, the present study also revealed that a considerable number of other patients whose hematopoiesis was normoblastic excreted an excessive amount of the derivatives, and at the same time, there was no interrelation between the amounts of the derivatives and various hematological findings. Grasbeck *et al.* have suggested that megaloblastic change must be a very late sign of folic acid deficiency, *i.e.* it is the consequence of an extreme degree of folic acid depletion, and that the depletion is common to a wide variety of disease states in which hematopoiesis is commonly normoblastic (13).

Raised urinary FIGLU has been reported to be noted in thyrotoxicosis, hemolytic anemia, Hodgkin's disease, congestive cardiac failure, intestinal malabsorption (11,12), in patients with skin disorders (18), and hepatic cirrhosis, or liver disease (17), and various neoplastic diseases (9,16,19). Similarly, raised urinary urocanic acid levels in various diseases have been observed as a consequence of disturbance of histidine breakdown in folate deficiency and in other conditions (9,15,24).

Although other determinations for folate deficiency such as the estimation of serum folate levels were not performed in this study, the results of the histidine loading test in hematological disorders are consistent with those in many reports. The evidence that the excessive combined excretion was found in 45 % of the 40 cases for FIGLU and in 40 % of the 25 cases for urocanic acid reasonably reflects either an absolute or relative deficiency of folate, or some abnormality of folate metabolism.

In the recent reports, more stress has been focussed on the relative importance of urocanic acid over FIGLU for the estimation of folate deficiency because of several experiences in which predominant excretion of the former was found in various diseases. Merritt *et al.* have reported two cases of megaloblastic anemia with abnormal urocanic acid excretion but normal amount of FIGLU (23). Bennett and Chanarin have demonstrated the excess excretion of urocanic acid in 88 % of a large number of patients with a variety of clinical disorders (4), and Mohamed and Roberts have reemphasized the value of the compound (25).

For explaining the appearance of this compound, Baldrige has found a reduction of the urocanase activity in the liver of rats deficient in folic acid (2). According to Chanarin, the administration of a small dose of folic acid resulted in reactivation of urocanase, which in turn caused the high urinary FIGLU excretion in place of the high level of existing urocanic acid (9). The present study also demonstrated the relative importance of urocanic acid excretion in urine. As compared with the report of Bennett and Chanarin (4) or of Mohamed and Roberts (25), the result of this study showed a less frequency in the appearance of excessive amount of urocanic acid. Ten patients out of 25 showed increased amount of urinary urocanic acid, and six patients out of these were seen to excrete combined FIGLU and urocanic acid in excess. Of these, the amount of urocanic acid exceeded that of FIGLU in one case with Hodgkin's disease, and the former alone in excess was found in half of six cases. These results would rather support the previous

report than minimize the importance of estimation of urocanic acid. Histidine derivatives have been reported not to be always responsive to folic acid or vitamin B₁₂ administration.

The ineffectiveness of folic acid for abolishing the abnormal excretion of histidine derivatives has been noted in a patient with idiopathic steatorrhea (25), or in patients with megaloblastic anemia and normoblastic haematopoiesis (9). Likewise, Chanarin has demonstrated that the therapy with vitamin B₁₂ did not change the abnormal excretion of histidine derivatives in a patient with folic acid deficiency (8), and in turn, Knowles and Pranker have found the ineffectiveness of folic acid therapy to reduce the derivatives in patients with vitamin B₁₂ deficiency (18).

However, the majority of the attempts with folic acid therapy have been found, in general, to produce a decline in the excretion of histidine derivatives. The effectiveness of folic acid therapy was recorded in various diseases including megaloblastic anemia of folic acid or vitamin B₁₂ deficiency (9,12,21), and other cases of normoblastic haematopoiesis, such as idiopathic steatorrhea, bronchial carcinoma (9) and liver cirrhosis (8). Luhby *et al.* found that the fall of histidine derivatives to normal or near normal excretion took place within 24-48 hours, but Chanarin showed within 72-96 hours or later (21).

In case 1, a significant decrease of histidine derivatives was achieved after a dose of folic acid, but a considerably large amount was still excreted. This fact would either support the action of folic acid abolishing histidine derivatives, or presumably suggest a diagnostic value for discrimination between folic acid and vitamin B₁₂ deficiency states. However, in this case the latter situation cannot be adaptable because the administered dose of folic acid was larger than that of the preceding reports, demonstrating that the therapeutic trial dose of the vitamin should be the order of 0.05 to 0.2 mg for ten days so as not to elicit a nonspecific reaction or a mass action (22,33). It is reasonable to assume that the decrease of histidine derivatives was attributed to nonspecific response in the case of this study. In the clinical examination to assess this response, no change could be found in megaloblast count of bone marrow, hemoglobin concentration or red blood cell count, and in the amount of methylmalonic acid excretion (see Fig. 4). Furthermore, subsequent therapeutic trial with coenzyme B₁₂ and CN-B₁₂ succeeded in abolishing the derivatives as well as inducing hematological response, which strongly supported the deficiency of vitamin B₁₂.

In case 2, the dose of folic acid administration was relatively smaller than that of case 1. Nevertheless, the increased urinary excretion of histidine derivatives responded to the folic acid therapy and markedly decreased to normal range, but the other examinations, especially that of morphological changes of bone marrow, revealed that the patient was of vitamin B₁₂ deficiency. This fact might probably suggest the too high order of dose of folic acid for differentiating the cause of deficiency, and simultaneously, the complexity in determining a proper dose of folic acid for the therapeutic trial.

In any way, folic acid appeared at least as effective as vitamin B₁₂ for abolishing the amount of histidine derivatives in pernicious anemia. Further investigation is needed to analyze the correlation between folic acid or vitamin B₁₂ and the response of histidine derivatives in urine.

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