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Detection and Isolation of Formiminoglutamic Acid from Urine in Folic Acid Deficiency in Humans.*† (24623)

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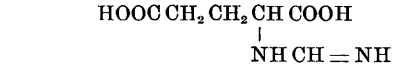
Silverman and coworkers(1) found that rats on folic acid (FA) deficient diet excreted in the urine a compound which was converted to glutamic acid when heated or exposed to alkali. Excretion of the glutamate precursor was associated with severity of FA deficiency (1) and increased by supplementing the diet with histidine(2). Subsequent chemical evidence(3) indicated that the glutamate precursor was N-formiminoglutamic acid (formamidinoglutaric acid):



Formiminoglutamic acid (FIGLU)

That a metabolic relationship between FA and FIGLU exists has become clearer with recent enzyme studies. Thus it has been shown that FIGLU may arise from degradation of histidine by liver homogenates and *Pseudomonas* extracts(4) and that tetrahydrofolic acid (THFA) functions in certain mammalian enzyme systems(5,6) as acceptor of formimino group of FIGLU giving formimino-THFA(6) and glutamic acid. Our study was undertaken to determine if FIGLU might be excreted in FA deficiency in man. Such findings would support concept that FA functions in formimino group transfer, and suggest detection of FIGLU in human urine be considered as a diagnostic tool for folic acid deficiency in man. Preliminary findings have previously been reported by us(7,8,9).

Methods. Twelve children with acute leukemia receiving various types of chemotherapy, one woman with macrocytic anemia of pregnancy and 5 normal subjects were studied. Highlights of clinical findings in addition to data in Table I, are: Subj. 1: A.B. Acute myeloblastic leukemia associated with mongolism, no previous antileukemic therapy, in clinical and hematologic relapse, blood WBC 28,600 mm^3, when urine collected; Subj. 2: S.M. Subacute lymphatic leukemia, no response to previous 6-mercaptopurine (Purinethal)® or steroid therapy, in clinical and hematological

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relapse, blood WBC 15,600 mm³, when urine collected; Subj. 3: R.F. Acute myeloblastic leukemia, excellent response to previous 6-mercaptopurine (Purinethal)® therapy followed by complete relapse; 6-MP discontinued 3 weeks previously, aminopterin 0.5 to 1.0 daily dose; moderate stomatitis, blood WBC 1,500 mm³, Subj. 4: D.A. Acute stem cell leukemia in clinical and hematologic remission with amethopterin (Methotrexate)® administered intermittently as maintenance therapy, received 105 mg prior to urine sample "d"; blood and marrow "practically" normal. Urine sample "e" collected 3 weeks later after amethopterin (Methotrexate)® discontinued because of stomatitis. *Urine for FIGLU isolation and identification* was collected from this patient in large quantities for several days after amethopterin resumed at average daily dose of 3.75 mg for several weeks, with patient still in clinical and hematologic remission. Subj. 5: M.R. Acute lymphoblastic leukemia, in clinical and hematologic remission, amethopterin 2.5 mg daily as maintenance therapy. Subj. 6: S.F. Acute stem cell leukemia, going into relapse despite continuance of amethopterin, 2.5 mg daily, following excellent clinical and hematologic remission. Subj. 7: W.M. Acute myeloblastic leukemia, no clinical or hematologic response despite 5 mg daily dose amethopterin when sample "h" collected; amethopterin discontinued because of stomatitis and leukopenia, blood WBC 1,600 mm³, when sample "i" collected. Subj. 8: J.P. Acute granulocytic leukemia, no clinical or hematologic remission although amethopterin administered 2.5 mg daily in first 8 weeks, 3.75 mg daily next 2 weeks and 5 mg last week. No mouth toxicity, blood WBC 2,000 mm³, marrow 93% "blasts" Subj. 9: M.S. Acute stem cell leukemia, in complete clinical and hematologic remission following 6-mercaptopurine (Purinethal)® regimen at 100 mg daily. Subj. 10: T. K. Acute micromyeloblastic leukemia, in clinical and hematologic relapse on 6-mercaptopurine regime of 37.5 mg daily. Subj. 11: T.B. Acute stem cell leukemia, in complete clinical and hematologic remission on 6-mercaptopurine regimen of 42.5 mg daily. Subj. 12: L.B. Acute stem cell leukemia, in partial hematologic remission on prednisone (Metacorten)® 60 mg daily. *Macrocytic anemia of pregnancy.* Subj. 13: M.A. discovered on first postpartum day, characterized by typical findings of peripheral blood macrocytosis, anemia (hematocrit 19%), leukopenia (WBC 3,000 mm³) and hypersegmented polymorphonuclear leukocytes. Bone marrow aspirate showed erythroid hyperplasia with typical megaloblastic features. On third day after diagnosis established, folic acid, 20 mg daily was administered orally for next 16 days. Patient's blood showed characteristic reticulocyte response reaching peaks of 13.5% on fifth and eighth day after start of folic therapy. When urine sample "p" collected, hematocrit had risen to 37.5%, WBC to 7,500 mm³. Bone marrow was normoblastic. *Control subjects* 14 to 18 incl. were normal individuals on average diets and showed no hematologic abnormalities. Several patients with iron deficiency anemia and Addisonian pernicious anemia were also studied as control subjects. Twenty-four hour *urine specimens* were collected under toluene in bottles containing sufficient glacial acetic acid to give final concentration of 1% and were held at 4°C, until prepared for assay. All urines were assayed within 7-14 days after collection. Upon arrival at laboratory, urine was adjusted to pH 6.5-7, clarified by filtration, and an aliquot sterilized by Seitz filtration. A portion of sterile filtrate was autoclaved 15 minutes. Urine specimens from individuals treated with aminopterin (4-amino-FA) or amethopterin (Methotrexate,® 4-amino - 10 - methyl - FA) were clarified by filtration and then stirred with 2% charcoal (Darco G-60) at pH 2-3 for 30 minutes, filtered and then sterilized as described above. The charcoal absorption served to remove FA antagonists which could interfere with the microbiological assay. *Microbiological assay* for glutamic acid using *Lactobacillus arabinosus 17-5* was employed following procedure of Henderson and Snell (10) and Hac *et al.*(11). Aliquots of *unheated* sterile urine and autoclaved (or *heated*) sterile urine were added aseptically to a series of 11 x 180 mm test tubes containing 1 ml of sterile, double strength, glutamic acid free medium(10) and the volume of

TABLE I. Microbiologic Evidence for Urinary Excretion of Formiminoglutamic Acid.

Subject	Clinical status: Age (yr)	Sex	Disease state	Therapy and duration	Sample No. and date	Urinary glutamate activity: Unheated aliquot	Heated aliquot	Ratio H/U
				Acute leukemia				
A.B.	2½	♂	Relapse	None	a. (2- 3-55)	49	14	.3
S.M.	10	♂	"	"	b. (4- 4)	140	50	.4
R.F.	8	♂	Partial remission	Aminopterin 11 days	c. (7-12)	78	109	1.4
D.A.	14	♀	Remission	Methotrexate* 2 wk	d. (6-14-56)	85	99	1.2
				After discont. 2 days	e. (7- 6)	35	10	.3
M.R.	5	♀	"	Methotrexate 4 wk	f. (7-17)	23	126	5.5
S.F.	3	♀	Relapse	Methotrexate 6 wk	g. (6-28)	140	304	2.2
W.M.	10	♂	"	Methotrexate 6 wk	h. (6- 5)	63	75	1.2
				After discont. 3 days	i. (6-11)	41	15	.4
J.P.	5½	♂	"	Methotrexate 8½ wk	j. (6-21)	74	144	1.9
M.S.	9	♂	Remission	Purinethal† 6 wk	k. (11- 7)	50	20	.3
T.K.	2½	♂	Relapse	Purinethal 2 wk	l. (")	51	<10	.2
T.B.	4	♀	Remission	Purinethal 13 wk	m. (")	64	41	.6
L.B.	10	♀	Partial remission	Metacorten‡ 3 wk	n. (11-12)	60	<10	.2
				Macrocytic anemia of pregnancy				
M.A.	22	♀	Post-partum 1 day	None	o. (12- 3-55)	77	99	1.3
			Post-partum 6 wk	Folic acid 16 days	p. (1-16-56)	98	19	.2
				Controls				
AHB	6	♂	Normal child		q. (7-12)	75	<10	.1
SAB	12	♂	*Idem*		r. (")	63	"	.2
HPB	Adult	♂	"		s. (")	125	"	.1
LAH	"	♂	"		t. (7-14)	99	"	.1
AMA	"	♀	"		u. (")	57	"	.2

* Amethopterin. † 6-mercaptopurine. ‡ Prednisone.

tubes was adjusted to 2 ml. The tubes were then inoculated with a "heavy inoculum"(11), and the assay incubated for 72 hours at 37°C. Growth was estimated turbidimetrically.

Presence of FIGLU in test sample was inferred from these considerations: Glutamic acid is fully active for *L. arabinosus* whether it is heated (autoclaving 15 minutes at pH 6.5-7) or added aseptically. Glutamine, on the other hand, is destroyed by this heating procedure and is active only if added aseptically. FIGLU cannot be utilized by *L. arabinosus* as a source of glutamic acid(12) but when it is autoclaved under conditions described above, it is hydrolyzed almost completely to glutamic acid (Luhby, A. L. and Cooperman, J. M. — Unpublished data). Growth response to *unheated* urine samples was regarded as a response primarily to glutamine for free glutamic acid content of normal urine is low relative to glutamine(13). Thus any significant growth response to *heated* urine samples was regarded as presumptive evidence for presence of FIGLU.

Results. Table I gives the glutamic acid excretion patterns of the subjects. Urine from

normal adults and children (subjects 14-18) contained ample glutamic acid activity when urine samples were not heated, but were very low in activity when heated. This ratio (H/U) is low in normal individuals. Urine specimens from children with acute leukemia, either untreated (subjects 1 and 2), or who were being treated with Purinethal® (subjects 9-11) or Metacorten® (subject 12) had H/U values similar to those of normal children. However, urine obtained from 6 children with acute leukemia who were on maintenance therapy with FA antagonists (subjects 3-8) increased in apparent glutamic acid content when heated (H/U ratio >1). Moreover in 2 instances (samples e and i) when the antagonist was withdrawn there was a concomitant decrease in the H/U ratio. Thus an increased H/U ratio is associated with FA antagonist therapy, but not with leukemia. Following administration of folic acid and complete hematologic and clinical remission, the H/U ratio (sample p) decreased to 0.2. A limited number of urine specimens from individuals with iron deficiency or Addisonian pernicious anemia have not shown evidence of FIGLU.

Isolation of glutamate precursor from urine. 3.1 liters of urine were collected from a 13-year-old child with acute leukemia in remission, Subject 4, while on maintenance therapy with amethopterin (*cf.* Experimental). Urine was clarified by filtration, adjusted to pH 2.5 with HCl, and stirred for 30 minutes with 31 g Darco G-60 activated charcoal. The charcoal was removed by filtration on Buechner funnel with the aid of Filtercell. An aliquot of the clear filtrate adjusted to pH 6.5 and autoclaved 15 minutes contained 180 μg/ml glutamic acid activity by microbiological assay. The charcoal filtrate was reduced to 1 liter by vacuum distillation at 25°C and the glutamate precursor isolated by ion exchange chromatography following the procedure of Silverman *et al.*(1). The concentrate was passed through a Dowex 50 (200 x 400 mesh, crosslink 8) H+ column (1½" wide packed to depth of 20") at 100 ml/hr., and 1 liter of water and 0.5 liter quantities of 1.5 μg and 10% acetic acid solutions passed successively through the column. The column was then placed on a fraction collector and developed with 1% sulfuric acid (flow rate, 50 ml/hr). Aliquots of all fractions were spotted on paper and sprayed with ninhydrin, or first exposed to ammonia vapor for 1 hour, aerated, then sprayed with ninhydrin. All fractions that were ninhydrin negative before ammonia treatment, but gave a color indicative of an α-amino acid after ammonia exposure, were pooled. 250 ml of such a pooled fraction was placed in a precipitating dish in icebath and 9 ml of 25% mercuric acetate in 0.2 M acetic acid was slowly added to form a mercury salt (12a). The precipitated mercury salt was collected at the centrifuge and washed successively with water, ethanol, and ether, air dried, to give 420 mg of nearly white material. An attempt was made to further purify this mercury salt by reprecipitation with mercuric acetate, with the glutamate derivative in excess to minimize contamination with mercuric oxide: 350 mg of mercury salt was suspended in 30 ml 0.01N sulfuric acid. Hydrogen sulfide was passed through it until precipitation of mercuric sulfide was complete. The latter was removed by centrifugation, and the clear filtrate vigorously aerated. Aliquots of this filtrate when chromatographed on paper had a major component indistinguishable in 2 solvent systems from synthetic FIGLU (Fig. 1). The remainder of the filtrate was treated with 1.3 ml mercuric acetate reagent as described above to finally give 165 mg of white mercury salt. Elemental analysis of this derivative was as follows:

$C_6H_{10}N_2O_6Hg_2$ (606)

Calculated:	C, 11.9; H, 1.7; N, 4.6
Found:	C, 10.5; H, 1.7; N, 4.3

Degradation products of isolated mercury salt. The mercury salt was subjected to various hydrolytic procedures shown in Table II. A mole of ammonia N was released/mole of mercury salt by either acid or alkaline hydrolysis. 0.7 mole of glutamic acid/mole of product was recovered by acid hydrolysis. When a cruder mercury salt was either autoclaved or let stand at pH 12 for 24 hours at 25°C, appearance of glutamic acid was demonstrated by paper chromatography(12). The chemical and microbiological properties de-

TABLE II. Products of Hydrolysis of Isolated Mercury Salt.

Method of hydrolysis*	Hg salt used, μM	Ammonia N,† μM	L-glutamic acid,‡ μM	Recovery for $C_6H_{10}N_2O_6Hg_2$, %
1 ml 4.5 N KOH, 120°C, 1.5 hr	20	18.6		93
1 ml 6 N HCl, " 15 "	"	19.3		97
Idem	"		14	70

* Hg salt suspended in indicated reagents in 1 ml ampul, the ampul sealed and autoclaved.
† Determined by Nesslerization.
‡ Hg^{++} removed from hydrolysate by precipitation as sulfide, and glutamic acid content of filtrate determined by microbiological assay(10).

scribed for the glutamic acid precursor isolated, indicate that it is a highly purified mercury salt of N-formiminoglutamic acid.

Discussion. It is now clear that in suitably purified mammalian enzyme systems(5,6), THFA is required for catabolism of FIGLU to glutamic acid.

One action of the 4-amino folic acid antagonists is to block one stage of the reduction of folic acid to THFA(14) as indicated in the

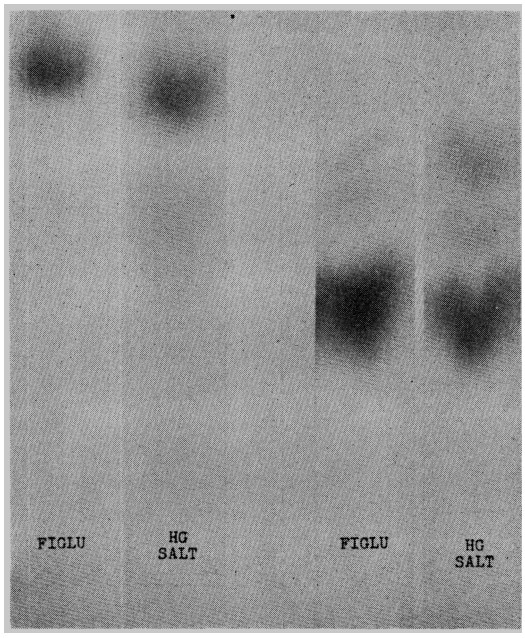

FIG. 1. Paper chromatography of synthetic formiminoglutamic acid (FIGLU) and glutamate precursor (mercury salt) isolated from urine. Solvent system first 2 chromatograms: collidine (1), lutidine (1), trimethylamine (1%), and water (1). Solvent system in last 2 chromatograms: n-butanol (40), acetic acid (10), water (50), used butanol layer. 50 γ synthetic FIGLU and .02 ml free acid of mercury salt (see text) were spotted at origin of 25 mm width Whatmann #1 paper strips, allowed to develop by ascending technic at 25°C for 40 hr, air dried, exposed to ammonia vapor 1 hr, aerated 1 hr, then sprayed with ninhydrin.

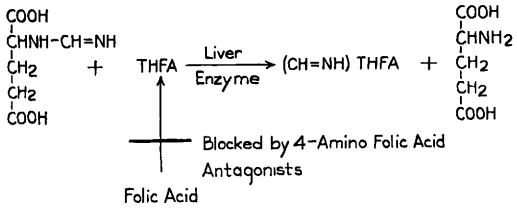

diagram. Hence the excretion of FIGLU in children receiving folic acid antagonists very likely occurs as a consequence of insufficient THFA essential for disposition of FIGLU. This substance might also accumulate in urine if the diet were deficient in folic acid or if pregnancy reduced maternal stores of folic acid. It is thus possible that determination of FIGLU in urine may be an aid in diagnosing nutritional folic acid deficiency.

Tabor and Wyngarden recently published an enzymatic method for determination of FIGLU in urine(15). With this method Hiatt *et al.*(16) also found that FIGLU is excreted in substantial quantities in children treated with amethopterin.

FIGLU, isolated as described above from human urine, was as active as an authentic sample of FIGLU kindly supplied by Dr. Herbert Tabor, when the natural and synthetic materials were compared in the enzymatic assay(15).

Summary. 1. A microbiological assay procedure giving presumptive evidence for presence of formiminoglutamic acid (FIGLU) was carried out on urine specimens from normal individuals, children with acute leukemia receiving various types of chemotherapy, and a patient with macrocytic anemia of pregnancy. The data indicated strongly that FIGLU was being excreted in children with acute leukemia receiving 4-aminofolic acid antagonists and in macrocytic anemia of pregnancy. 2. A

highly purified mercury salt of a glutamic acid precursor was isolated from urine of patient with leukemia during amethopterin therapy, and on the basis of its chemical and microbiological properties, concluded to be FIGLU. 3. It is postulated that folic acid antagonists induced a folic acid deficiency, resulting in insufficient tetrahydrofolic acid needed for catabolism of FIGLU. Consequently FIGLU accumulated. It is suggested that determination of FIGLU in human urine may be a useful aid in diagnosing folic acid deficiency in man.

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Effect of Food Deprivation on Thermal Behavior of the Rat. (24624)

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Ordinarily the laboratory rat has little or no control over his environmental temperature. When cold stressed, this organism, unlike man, does not have access to external heat control devices. Recently McCleary *et al.* (unpublished) investigated behavioral results of providing the rat with some control over environmental temperature. Rats were exposed to low ambient temperature and given a response pedal, which when depressed, activated a 10 second burst of heat from an infra red heat lamp. Under these conditions the rate of bar pressing was shown to be a function of ambient temperature. Weiss(1) has extended this work to show that the bar press rate under above conditions is also a function of restricted food intake and pantothenic acid deprivation. The present study is a further extension of the above work, using a modified dependent variable with 3 levels of food deprivation.

Method. Six adult male Sprague Dawley rats were used. The experimental apparatus was a rectangular box of galvanized steel, 15 by 24 by 10 inches. A 3/8 inch round response bar protruded into the box 4¼ inches and was located 4½ inches in front of 2 GE 250 watt infra red heat lamps with built-in red filters. The lamps were separated from the bar compartment by a partition of ¼ inch hardware cloth. Depression of the bar closed a microswitch and activated the heat lamps. At the same time a cumulative timer and response counter were energized. This apparatus was placed in a conventional refrigerator with ambient temperature at 4° ± 1°C at beginning of experimental session. Auxiliary lighting was supplied by 2 NE 40 GE glow lamps at top of side opposite the heat lamps. Preliminary training of animals consisted of exposure to experimental conditions for 2 hours before first day of recording. The animal's coat was clipped and it was placed in the apparatus and left to discover the response bar and its func-