A METHOD FOR THE DETERMINATION OF FORMIMINOGLUTAMIC ACID IN URINE [1]

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Formimino-L-glutamic acid, an intermediate in the metabolism of histidine (1, 2), accumulates in the urine of folic acid-deficient rats (3–7). The urinary formiminoglutamic acid has also been reported to be increased in macrocytic anemia of pregnancy and in patients treated with folic acid antagonists (8–10). These findings can be attributed to a lack of tetrahydrofolic acid (THF), since enzymatic studies have shown (11–14) that THF is required for the further metabolism of formiminoglutamic acid.

The accumulation of formiminoglutamic acid in the urine can be used as a test for folic acid deficiency both in rats and in patients. The available assay for formiminoglutamic acid, based on the microbiological assay of the glutamic acid formed from formiminoglutamic acid by heating (4, 9), is relatively inconvenient and inaccurate. The assay is complicated by the fact that glutamic acid and glutamine are normally present in urine, as well as by the variable conversion of these compounds to pyrrolidone carboxylic acid during the heating step.

In this paper a sensitive spectrophotometric method is presented for the assay of formimino-L-glutamic acid in the urine. This method is based on enzymatic reactions (1) and (2) [Diagram 1] which we have recently (14) demonstrated to be involved in the metabolism of formiminoglutamic acid.

The amount of 5,10-methenyl-THF formed in reaction (2) is easily measured spectrophotometrically, since this compound has a high absorption in the ultraviolet with an extinction coefficient of 25,000 at 350 mμ (15–18). During the course of the reaction, however, 5,10-methenyl-THF is converted nonenzymatically to 10-formyl-THF in accordance with reaction (3).[2] The latter compound, however, is completely reconverted to 5,10-methenyl-THF upon acidification.

In the quantitative procedures presented here,[3] urine is incubated in phosphate buffer with a mixture of the transferase, cyclodeaminase and tetrahydrofolic acid. The mixture is then acidified, and the concentration of 5,10-methenyl-THF is calculated from the optical density at 350 mμ.

EXPERIMENTAL

Enzyme preparation. Commercial frozen hog liver is used as the starting material; this can be stored at $-20°$ C. for as long as six months without loss of activity.

Step A. For the preparation of "acetone powders," 100 gram portions of hog liver are homogenized in a Waring blendor with approximately 500 ml. of acetone (technical grade) at room temperature for one minute; the mixture is then filtered rapidly through a large Büchner funnel. The moist filter cake is homogenized again with acetone, refiltered, passed through a coarse screen, and dried at room temperature overnight. The resultant powder can be stored over a drying agent *in vacuo* at $-20°$ C. for as long as six months.

Step B. Extracts are prepared by gently stirring 160 grams of acetone powder with 1,600 ml. of water at room temperature. After 15 minutes the suspension is centrifuged in an angle centrifuge at $20,000 \times G$[4] for 15 min-

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[2] The nonenzymatic conversion of 5,10-methenyl-THF to 10-formyl-THF at pH 7 is catalyzed by the phosphate buffer. This reaction is also rapidly catalyzed by the enzyme cyclohydrolase (14, 16, 17). This activity is not present, however, in the partially purified preparations described in this paper.

[3] Silverman, Gardiner, and Condit (19) have recently developed a method for formiminoglutamic acid estimation which depends on the formation of 10-formyl-THF from formiminoglutamic acid and folic acid in a crude chicken liver homogenate. The latter contains the enzymes mentioned above plus a folic acid reductase. In this precedure the 10-formyl-THF is converted to 5-formyl-THF by heating, and the latter is assayed microbiologically.

[4] Lower centrifugal speeds are satisfactory at this point.

(1)

NH
‖
CH
|
NH
|
HOOC–CH–CH₂CH₂COOH

+

[tetrahydrofolic acid structure] CH₂–NH–R

FIGLU
TRANSFERASE

FORMIMINOGLUTAMIC ACID
(FIGLU)

TETRAHYDROFOLIC *
ACID
(THF)

NH₂
|
HOOC–CH–CH₂CH₂COOH + [5-formimino-THF structure] CH₂–NH–R

GLUTAMIC ACID   5–FORMIMINO–THF

(2)

[5-formimino-THF structure] CH₂–NH–R

FORMIMINO–THF
CYCLODEAMINASE
OR ACID

[5,10-methenyl-THF structure] CH₂–N⁺–R + NH₃

5–FORMIMINO–THF   5,10–METHENYL–THF

* R = [benzene ring] CONHCH–CH₂CH₂COOH ,
            COOH

(3) [5,10-methenyl-THF structure] CH₂–N⁺–R + H₂O ⇌ OH⁻ / H⁺ [10-formyl-THF structure] CH₂–N–R / CHO + H⁺
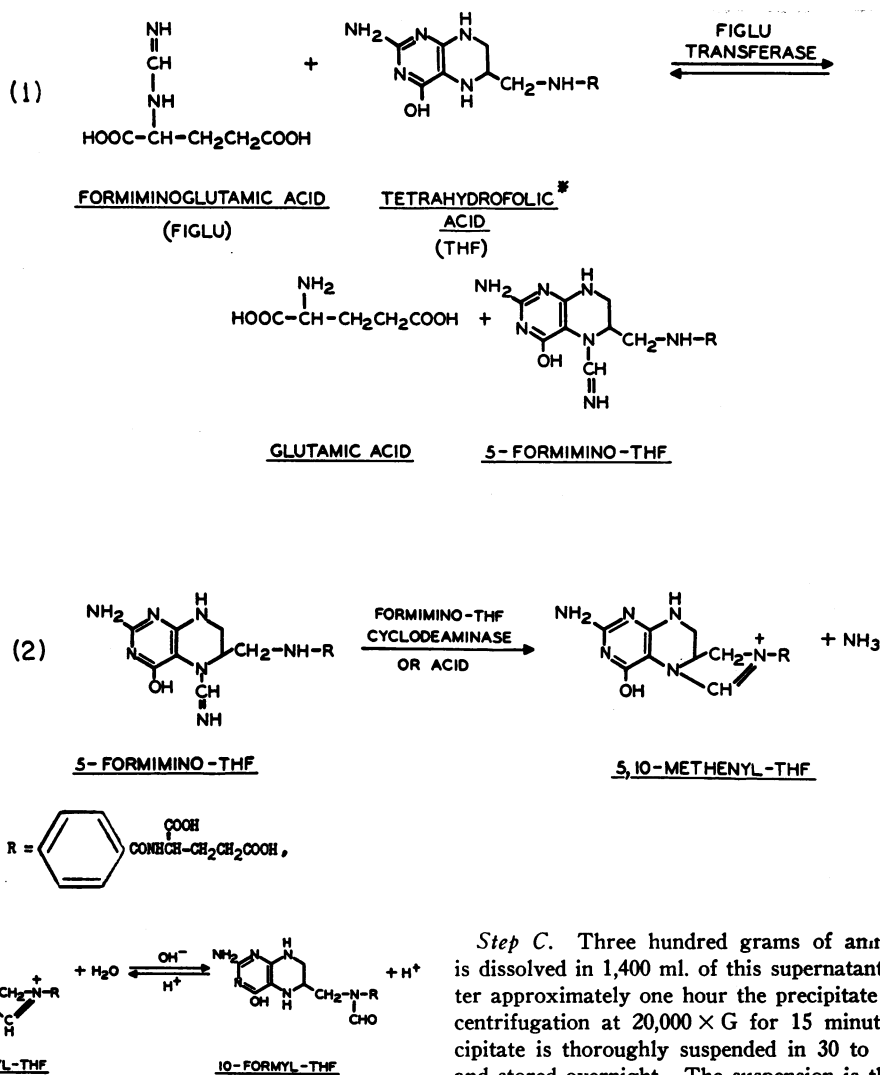
5,10–METHENYL–THF   10–FORMYL–THF

DIAGRAM 1

utes, and the supernatant solution collected.[5] Subsequent steps are carried out at 1 to 3° C.

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[5] The enzyme purification procedure presented here has been very reproducible, and enzymatic assays have not ordinarily been carried out after each step. If desired, however, the following rapid assay can be used to follow the purification procedure. Although more precise assays for each enzyme activity are available (to be published), they are not necessary for the purposes of this paper.

The assay mixture is similar to that described below under "assay," and contains 0.1 ml. of 1 M potassium phosphate buffer (pH 7.2), 0.1 ml. of the tetrahydrofolic acid–mercaptoethanol solution, 5 micromoles of sodium formiminoglutamate, enzyme solution, and water to 1 ml. After five minutes at 25° C., 0.3 ml. of 10 per cent perchloric acid is added. The mixture is heated for one-half to one minute in a boiling water bath, cooled im-

Step C. Three hundred grams of ammonium sulfate is dissolved in 1,400 ml. of this supernatant solution. After approximately one hour the precipitate is collected by centrifugation at 20,000 × G for 15 minutes.[4] The precipitate is thoroughly suspended in 30 to 50 ml. of H₂O and stored overnight. The suspension is then centrifuged for two hours at 30,000 × G in an angle centrifuge, and the supernatant solution is discarded. The precipitate is then dissolved in 38 ml. of 0.2 M sodium acetate; after four hours the solution is centrifuged, and any insoluble material is discarded. This enzyme fraction is used for the assay procedure.

This preparation contains both the transferase and the cyclodeaminase. It has been stored with little loss of activity at − 20° C. for over six months, at 0° C. for over

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mediately in ice water, centrifuged, and the optical density of the supernatant solutions measured at 365 mμ against a suitable blank without formiminoglutamic acid. Under these conditions 0.01 ml. of this extract results in an increase in optical density of 0.6 to 1.0.

The same assay procedure can be used to follow Step C, using suitable dilutions of the enzyme. Step C results in a purification of approximately one hundredfold with about a 50 per cent yield of both the transferase and the cyclodeaminase.
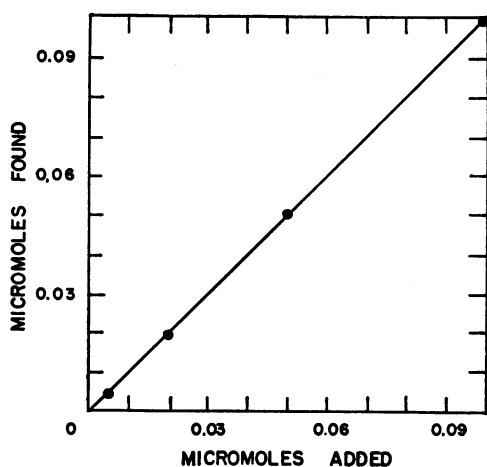
FIG. 1. RECOVERY OF FORMIMINOGLUTAMIC ACID ADDED
TO URINE

Varying quantities of formiminoglutamic acid were
added to 0.45 ml. aliquots of human urine. The mixture
was then assayed for the formiminoglutamic acid content.

three months, and as a lyophilized powder at −20° C. for
over three months. Details on the further purification and
separation of these enzymes will be published separately.

Tetrahydrofolic acid. Folic acid is reduced by the
method of Jaenicke and Greenberg, as modified by Rabino-
witz and Pricer (18). Solutions are prepared by dis-
solving 88 mg. of tetrahydrofolic acid in 18 ml. of 1.1 M
mercaptoethanol plus 2 ml. of 0.2 N KOH. These solu-
tions are usually 70 to 80 per cent pure, and contain 7 to
8 micromoles of dl-THF per ml. They are stored in
test tubes *in vacuo* in the dark at 0° C., and have been
stable for at least three months. Aliquots are trans-
ferred to small test tubes for daily use. The synthetic
procedures used for the preparation of *formiminoglutamic
acid, formiminoaspartic acid,* and *formylglutamic acid*
have been described previously (2, 20).

Assay procedure. Urine (containing less than 0.1 mi-
cromole of formiminoglutamic acid), 0.25 ml. of the en-
zyme preparation, 0.1 ml. of tetrahydrofolic acid solution,
0.1 ml. of 1 M potassium phosphate buffer (pH 7.2), and
water up to 1 ml. are incubated in narrow 3 ml. test
tubes at 25° C. for 30 minutes. A "control" mixture is
prepared in the same way except for the omission of
enzyme. At the end of the incubation period 0.3 ml. of
10 per cent perchloric acid [6] is added; after two to two
and one-fourth hours the solutions are centrifuged,[7] and
the optical density at 350 or 365 mμ is read against the

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[6] The perchloric acid is added both to deproteinize the
incubation mixture and to convert formiminotetrahydro-
folic acid and 10-formyltetrahydrofolic acid to 5,10-
methenyl-THF.

[7] Alternatively, the mixture, after the addition of per-
chloric acid, is heated for one-half to one minute in a
boiling water bath, cooled immediately in ice water,
centrifuged, and the optical density of the supernatant
solutions measured.

"control" cuvette. If the optical density is too high, the
readings can be taken at 380 mμ where the extinction
coefficient is lower. The optical density readings were
corrected for a small enzyme blank (0.03 to 0.09). This
value was obtained by incubating buffer, enzyme and
THF; after the addition of perchloric acid (as above),
the optical density was read against a similar mixture
without enzyme.

The spectrophotometric readings are made in semi-
microcuvettes (1.0 to 1.5 ml. volume) with a 1 cm. light
path. The optical density obtained with 0.01 micromole
of formiminoglutamic acid in 1.3 ml. volume under these
conditions is 0.19 at 350 mμ, 0.17 at 365 mμ, or 0.11 at
380 mμ. Therefore, the quantity of formiminoglutamic
acid in micromoles in the aliquot used is calculated by
multiplying the corrected optical density by 0.052 at 350
mμ, 0.059 at 365 mμ, or 0.088 at 380 mμ. The sensitivity
of the procedure is approximately 0.001 micromole.

Recovery of formiminoglutamic acid from urine. Good
recovery is obtained when varying quantities of formimi-
noglutamic acid are added to water or to urine (Figure
1). Duplicate assays usually agree within 0.001 micro-
mole, even when the determinations are carried out with
different enzyme and tetrahydrofolic acid preparations.
No inhibitory effects are observed with rat urine or with
human urine, even after amethopterin (methotrexate)
treatment (21).

Specificity. With the assay conditions used, no reac-
tion (< 0.002 micromole) is obtained with 10 micro-
moles of L-methionine, L-serine, sodium formate, L-histi-
dine, ammonium chloride, L-glutamic acid, formamide,
formiminoglycine or glycine.

The enzyme preparation reacts very slowly with both
formylglutamic acid (13, 22) and with formiminoaspartic
acid. With 1 to 10 micromoles of either formylglutamic
acid or formiminoaspartic acid, a reading equivalent to
approximately 0.06 micromole of formiminoglutamic acid
is obtained. There is no evidence at present that urine
contains either formiminoaspartic acid or formylglutamic
acid.

The enzyme preparation still has traces of urocanase.[8]
However, even with 10 micromoles of urocanic acid, a
maximum of 0.013 micromole of formiminoglutamic acid
is formed. Thus, no significant error is introduced in the
urinary assays at the levels of urocanic acid usually found
in urine. Any error due to urocanic acid can be detected
by the alkaline hydrolysis procedure described below,
since urocanic acid is relatively stable under these condi-

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[8] The enzyme preparation contains approximately 0.3
per cent of the urocanase activity found in the crude ex-
tract. A preparation entirely free of urocanase can be
obtained by treating 20 ml. of the enzyme solution with
2.0 ml. of a 3 M sodium acetate buffer [NaAC/HAc =
90/10] at 0° C. After four hours the precipitate is col-
lected by centrifugation at 30,000 × G for 30 minutes, and
dissolved in 20 ml. of 0.1 M triethanolamine sulfate buf-
fer (pH 7.1). This preparation represents a one thou-
sandfold purification of both the transferase and the
cyclodeaminase.

tions (> 95 per cent remaining after autoclaving for two hours).

Although no additional procedures have been used routinely for the further identification of the formiminoglutamic acid in urine, several procedures are available for this purpose:

1. *Alkaline hydrolysis.* Formiminoglutamic acid is very unstable at an alkaline pH. It has a half-life at 25° C. of 10 minutes in 1 N KOH, 20 minutes at pH 11.5, 70 minutes at pH 10.8, and 40 hours at pH 9.2. To destroy the formiminoglutamic acid, therefore, the urine is adjusted to pH 11.5 with KOH and incubated at 25° C. for four hours. This alkali treatment causes a decrease in the assay of over 97 per cent; the small residual reading is due to formylglutamic acid which accumulates during the mild alkaline hydrolysis, and, as indicated above, reacts slowly in the enzyme assay. To obtain complete hydrolysis, the following conditions are used. Two ml. of urine is neutralized with KOH; 0.1 ml. of 6 N KOH is then added, and the mixture is autoclaved for two hours at 15 pounds pressure. We have observed complete hydrolysis under these conditions in all the urine samples that we have tested.

Formiminoglutamic acid is very stable in acid at 25° C., showing no destruction after several months in 0.1 N acetic acid, 0.1 N HCl, and 6 N HCl. At pH 7 it is relatively stable at 0° C., but is destroyed (> 98 per cent) by autoclaving for two hours · (4).

2. *Alkaline ferricyanide-nitroprusside reaction.* Formiminoglutamic acid in urine may also be determined colorimetrically by a modification of the method used by Rabinowitz and Pricer for formiminoglycine determination (23, 24). Urine plus H_2O (0.5 ml. volume) is mixed with 2 ml. of saturated sodium borate and 0.5 ml. of the ferricyanide-nitroprusside reagent (4 grams each of NaOH, potassium ferricyanide, and sodium nitroprusside in 120 ml. of H_2O). The optical density at 485 mμ is read after 30 minutes at room temperature against a corresponding blank. The latter is prepared by preincubating 0.3 ml. urine with 0.1 ml. of 2.5 N KOH for 2 hours at 25° C. to destroy the formiminoglutamic acid present; the mixture is then neutralized with 0.1 ml. 2.5 N HCl, and assayed by the above procedure.

Under these conditions an optical density of 0.60 is obtained with 1 micromole of formiminoglutamic acid. Thus, this method is considerably less sensitive than the enzymatic procedure. The colorimetric assay has been satisfactory, however, for following the excretion of formiminoglutamic acid in the urines of folic acid-deficient rats, in which large amounts of this material are excreted.

3. *Chromatography.* Aliquots of urine containing large quantities of formiminoglutamic acid can be chromatographed on Whatman No. 1 paper in *t*-butanol 70, formic acid 15, H_2O 15 ($R_f = 0.52$) (2, 20, 25) or in the phenolphosphate-citrate solvent of Broquist (9). After removal of the solvent, the paper is placed in an atmosphere of ammonia for two hours to hydrolyze the formiminoglutamic acid (20). The ammonia vapors are removed by aeration; the paper is sprayed with a ninhydrin solution, and the color is developed at room temperature. A

control paper is usually run without the ammonia treatment, since formiminoglutamic acid gives only a very weak spot with ninhydrin under these conditions.

Formiminoglutamic acid may also be chromatographed on Dowex 50 and Dowex 1, as previously described (2, 4, 20).

DISCUSSION

Formiminoglutamic acid has been shown to accumulate in the urine in folic acid deficiency (3–10). A quantitative determination of urinary formiminoglutamic acid is useful for following the excretion of this compound in folic acid-deficient animals (3–7), in patients treated with anti-folic acid compounds (9, 10), and in certain macrocytic anemias (8).

The method presented here affords a simple, specific spectrophotometric procedure for the determination of formiminoglutamic acid, which is applicable to studies on its excretion in urine. We have recently used this method for following the excretion of formiminoglutamic acid in the urine during the development of a folic acid deficiency in rats; this was induced by feeding a folic acid-deficient diet, containing succinylsulfathiazole. These data, which will be reported in greater detail elsewhere (7), are summarized in Table I.

In the accompanying paper (21) the use of this procedure to assay the formiminoglutamic acid concentration in various human urines has been described.

SUMMARY

A sensitive spectrophotometric method is presented for the assay of formiminoglutamic acid in the urine.

TABLE I

Effect of folic acid deficiency on formiminoglutamic acid excretion

Day of diet*	Folic acid administered	Formiminoglutamic acid excreted
	0.05 µM/rat/day	*µM/7 hr. period*
7	0	0.22
14	0	1.29
21	0	2.06
28	0	9.66
35	0	21.2
41	0	36.5
43	+	0.38
45	+	0.18

* A folic acid-deficient diet, containing 1 per cent succinylsulfathiazole, was fed to six weanling rats. Further details on this experiment will be published subsequently in a more detailed study of the urinary changes in folic acid deficiency (7).

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