Metabolism of Formiminoglutaric Acid by Vitamin B₁₂ and Folic Acid-deficient Rats Fed Excess Methionine

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The amidine carbon atom (C-2) of L-histidine is known to be an efficient metabolic precursor of single carbon units. Following the administration of L-histidine-2-C¹⁴, the radioactivity has been found in CO₂ and urea, in carbons 2 and 8 of purines, and in the hydroxymethyl group of serine, and in choline, and creatine (1-4). The major pathway of histidine degradation which involves deamination to urocanic acid and finally, cleavage of the ring to formiminoglutamic acid (5-7) has been assumed to be the sole means of liberation of this carbon atom for "1 carbon metabolism." The liberation of the single carbon unit can be accomplished by transfer of the formimino group to tetrahydrofolic acid. The formimin moiety is ultimately transformed into the formyl group of N⁵-formyltetrahydrofolic acid (8, 9).

Folic acid or vitamin B₁₂-deficient rats excrete large amounts of formiminoglutaric acid in the urine (10, 11). In the more recent study (11), the basal diet employed was limiting in the sulfur amino acids (9% casein). The addition of excess methionine to the diet, in the absence of either folic acid or vitamin B₁₂, significantly reduces urinary formiminoglutaric acid (11). The studies reported in this paper were prompted by this finding and were designed to clarify the nature of this effect. These studies show that radioactive CO₂ (derived from carbon 2 of histidine) is greatly increased following the administration of excess methionine to the deficient animal. Furthermore, the evidence presented is consistent with the view that methionine does not divert histidine metabolism from the urocanic acid pathway, but exerts its influence at the level of formiminoglutaric acid.

EXPERIMENTAL PROCEDURE

Materials and Methods

Diets used (11) were modified to contain 0.2% added L-histidine monohydrochloride and 100 μg of vitamin B₁₂ per kg. Male rats of the Sprague-Dawley strain were fed the experimental diets after weaning. Uniformly labeled radioactive L-histidine-C¹⁴ (0.21 μc per μmole) was purchased from Schwarz Laboratories; L-histidine-2-C¹⁴ (3.56 μc per μmole), sodium formate-C¹⁴ and L-serine-β-C¹⁴ were purchased from Nuclear-Chicago. They were used without further purification. Uniformly labeled C¹⁴- and 2-C¹⁴-urocanic acid were formed enzymatically from their respective histidines (12) without dilution of specific activity. They were purified by fractionation on Dowex 1-acetate columns with gradient elution; the eluant was prepared by allowing 0.5 N acetic acid to drip into a reservoir containing 200 ml of water (13). No radioactive contaminants in these preparations could be detected by the isotope dilution technique (14), or paper chromatography with radioautography.

Dimethylacetothetin was the gift of Dr. W. Klee. Other compounds were obtained commercially.

Collection of Respiratory CO₂—Rats treated by injection intraperitoneally with histidine-C¹⁴ or urocanic acid-C¹⁴ contained in a volume of 0.5 to 1.0 ml were placed in a metabolic chamber and the CO₂ collected for 4 hours in 50 ml of 10% NaOH. The exact volume was recorded and aliquots assayed in duplicate for radioactivity, with a modification of the method of Passmann et al. (15). For each determination an aliquot (0.2 ml) of the above absorbing solution was pipetted into a single armed Warburg flask (14/26 joint), the mouth of the main compartment was fitted with an air tight rubber sleeve injection stopper, and the side arm was stoppered with a small cork following the addition of 0.5 ml of hyamine (15). With the flask sealed, 1 ml of 5 N H₂SO₄ was injected through the rubber stopper and the flask gently shaken for 2 hours. The absorption of CO₂ in the hyamine was complete in 60 minutes. The hyamine was then transferred to a counting vial and the side arm rinsed three times with 0.5 ml of toluene. These washings were added to the vial with 10 ml of phosphor and the solution counted in a Tri-Carb liquid scintillation spectrometer. Duplicate samples generally agreed within 10%. If there was more than 20% disparity, the radioactivity in the NaOH solution was re-assayed in duplicate.

After the injection of histidine-2-C¹⁴, radioactivity in the respiratory CO₂ reaches a peak between 1 and 2 hours, and about 50% of the cumulative 24-hour C¹⁴O₂ is exhaled in the first 4 hours after injection.

Assay of Urinary Metabolites—Formiminoglutaric acid: This compound was determined by a previously employed procedure (16). The volumes of reactants were reduced to one-fourth.

Formiminoglutaric acid-C¹⁴: The assay for this metabolite has been described (13, 14), and consists of determining the C¹⁴ lost after the urine sample is heated in NH₄OH and then in formic acid. Essentially only the C¹⁴ from the formimino group is volatilized under these conditions; less than 5% of the loss in activity is contributed by C¹⁴ from carbonate and formic acid in the samples.

Hydantoin propionic acid-C¹⁴: About 50 mg of carrier L-hydantoin-5-propionic acid were added to 5 ml of urine. This solution was then passed through a Dowex 50-H⁺ column (10 ×...
RESULTS

It was of primary interest to determine whether or not the action of methionine in reducing the excretion of formiminoglutamic acid was associated with the release of carbon 2 of histidine as a "C-1 unit." The urine from a folic acid- and a vitamin B12-deficient rat, both of which had been fed their respective diets for 5 weeks after weaning, was collected for 18 hours. The formiminoglutaric acid was determined (16) and the values were corrected for 24 hours. Immediately following the urine collection, the two deficient rats were injected "in the morning" with 3.1 £ (0.87 umole) of L-histidine-2-Cl4 and the respiratory CO2 was collected for 4 hours and assayed for C14 (Fig. 1). This sequence of urine collections, histidine injections, and CO2 collections was repeated for a period of 28 days. After the CO2 collection of the second day, the diets were supplemented with 1% L-methionine. Following the administration of this amino acid, there was a decrease in the excretion of formiminoglutaric acid and an increase in the respiratory C14O2. After the CO2 collection on the ninth day, the excess methionine was removed from the diet. The radioactive CO2 values returned to low levels and the urinary formiminoglutaric acid increased. On the 22nd day, the rats were treated by injection intraperitoneally with a solution of 50 mg of L-methionine 15 minutes before the injection of the radioactive histidine. There was a marked increase in C14O2 and a significant drop in urinary formiminoglutaric acid. By the following day, the C14O2 and formiminoglutaric acid values had returned to basal levels. Subsequent "preinjections" of 50 mg of L-homocysteine, glycine, or L-serine were without effect on the respiratory C14O2. Homocysteine, however, did decrease the urinary formiminoglutaric acid of both rats whereas serine caused an increase in the folic acid-deficient rat. The marked variability in C14O2 excretion during the period in which excess methionine was added to the diet can probably be explained by the levels of circulating methionine at the time of radioactive histidine injection. Since the fate of carbon 2 of histidine is so radically altered by a "preinjection" of methionine, it can be assumed that rats which had ingested larger amounts of the diet containing excess methionine just before radioactive histidine injection would convert more of the carbon 2 to CO2.

A vitamin B12-deficient rat excreting formiminoglutaric acid was fed a diet supplemented with 1% L-methionine for 2½ weeks before the initiation of a similar study. The results shown in Fig. 2 demonstrate that there is a marked "methionine effect." After the second day, the excess methionine was removed from the diet and the C14O2 dropped to low levels. Following the CO2 collection on the fourth day, supplementation of the diet with vitamin B12 caused a reversal of the deficiency. This is demonstrated by an increase in C14O2 and a decrease in urinary formiminoglutaric acid. At days 14 and 15 when there was no longer any demonstrable deficiency, the rat was treated by "preinjection" with 25 and 50 mg of L-methionine, respectively. Instead of the stimulation of C14O2 excretion that occurs when deficient rats are given methionine, there was a slight decrease. This depression of radioactive CO2 production in normal rats was verified in subsequent experiments.

Localization of the Methionine Effect—A study in vivo was done to localize the step at which methionine influenced histidine metabolism (Table I). On the first day, two vitamin B12-deficient rats which were excreting about the same amount of formiminoglutaric acid were treated by injection with urecanic acid-2-C14 and histidine-2-C14, respectively. On the second day, each rat was treated by injection with 50 mg of L-methionine 15 minutes before another injection of the radioactive urecanic acid or histidine. Urine was quantitatively collected under toluene for each 24-hour period and analyzed for total C14 content, formiminoglutaric acid-C14, and hydantoin propionate-C14. The above sequence of injections was repeated several days later and CO2 collections were made. The total urinary C14 is markedly decreased by methionine. Moreover, this decrease can be attributed largely to a reduction in formiminoglutaric acid, since the values for total urinary radioactivity minus radioactive formiminoglutaric acid are virtually unaltered by injection of methionine. Methionine must then be exerting its effect somewhere along the urecanic acid pathway.

When, several days later, the sequence of injections was repeated using uniformly labeled urecanic acid or histidine and the total radioactivity of the urine was measured, it became apparent that methionine depressed the urinary radioactivity of both types of labeled urecanic acid or histidine to approximately
than 0.3% of the injected radioactivity. There was no evidence for increased values in the urines of rats "pretreated" with methionine. Instead, the other 5 carbons of formiminoglutamic acid are also metabolized. Hydantoin propionic acid is formed by an enzymatic oxidation of 4(5)-imidazolone-5(4)-propionic acid (14), the biological intermediate between urocanic and formiminoglutamic acids. Only a small amount of the hydantoin propionic acid formed should be decreased. Since the per cent of the injected dose of either histidine or urocanic acid that is excreted as hydantoin propionic acid (Table I) is not greatly altered under conditions where formiminoglutamic acid excretion is markedly reduced, it can be assumed that methionine does not alter the urocanic acid pathway at the levels of urocanic acid or imidazolone propionic acid. Thus, methionine appears to influence histidine and urocanic acid metabolism at the formiminoglutamic acid level. Attempts to measure the amount of free glutamic acid-C$^{14}$ in the urine of rats given the uniformly labeled C$^{14}$ compounds were complicated by the slight hydrolysis of the 2-C$^{14}$ compound. Formiminoglutamic acid-C$^{14}$ in the sample. However, values for glutamic acid-C$^{14}$ obtained by the addition of carrier glutamic acid-U$^{14}$ in the urine of rats given the uniformly labeled Cl$^{14}$ were less than 0.3% of the injected radioactivity. There was no evidence for increased values in the urines of rats "pretreated" with methionine.

Table I

<table>
<thead>
<tr>
<th>Rat</th>
<th>Compounds injected*</th>
<th>Values are expressed as per cent of injected radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total radioactivity</td>
<td>Formiminoglutamic acid</td>
</tr>
<tr>
<td>A</td>
<td>Urocanic acid-2-U$^{14}$</td>
<td>79.4</td>
</tr>
<tr>
<td>A</td>
<td>Urocanic acid-2-C$^{14}$ + L-methionine</td>
<td>38.7</td>
</tr>
<tr>
<td>B</td>
<td>L-Histidine-2-C$^{14}$</td>
<td>27.8</td>
</tr>
<tr>
<td>B</td>
<td>L-Histidine-2-C$^{14}$ + L-methionine</td>
<td>14.9</td>
</tr>
<tr>
<td>B</td>
<td>Urocanic acid-U-C$^{14}$</td>
<td>67.7</td>
</tr>
<tr>
<td>B</td>
<td>Urocanic acid-U-C$^{14}$ + L-methionine</td>
<td>34.6</td>
</tr>
<tr>
<td>A</td>
<td>L-Histidine-U-C$^{14}$</td>
<td>23.3</td>
</tr>
<tr>
<td>A</td>
<td>L-Histidine-U-C$^{14}$ + L-methionine</td>
<td>9.3</td>
</tr>
</tbody>
</table>

* Urocanic acid-2-U$^{14}$ (2.3 μc, 0.65 μmole), L-histidine-2-C$^{14}$ (2.3 μc, 0.65 μmole), urocanic-U-C$^{14}$ (0.7 μc, 3.3 μmole), and L-histidine-2-C$^{14}$ (0.7 μc, 3.3 μmole) were injected intraperitoneally. Fifty milligrams of L-methionine dissolved in 1 ml of H$_2$O were used.

† The values given refer to the C$^{14}$ content of metabolites in urine collected for the 24-hour periods after the first set of injections of each labeled compound and to respiratory CO$_2$ collected for the 4-hour periods after the second set of injections of the 2-C$^{14}$ compounds made after an interval of several days.

Table II

<table>
<thead>
<tr>
<th>Days after initiation of diets</th>
<th>Compounds injected</th>
<th>Per cent of injected dose excreted as CO$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Histidine</td>
<td>9.9</td>
</tr>
<tr>
<td>4</td>
<td>Methionine + histidine</td>
<td>8.0</td>
</tr>
<tr>
<td>10</td>
<td>Histidine</td>
<td>8.1</td>
</tr>
<tr>
<td>11</td>
<td>Methionine + histidine</td>
<td>7.0</td>
</tr>
</tbody>
</table>

* Formiminoglutamic acid first was detected in the urine on the 7th day (excretion, 2.5 μmoles per day), and on the 13th day the rat excreted 14.4 μmoles of the formimin compound. L-Histidine-2-C$^{14}$ (1 μc 0.28 μmole), and L-methionine, 25 mg, were injected as described in the text.

Onset of the Methionine Effect—Weanling rats were placed on control and vitamin B$_{12}$-deficient diets. On the third day, CO$_2$ collections were made after the injection of 1.0 μc (0.28 μmole) of L-histidine-2-C$^{14}$. The following day, 25 mg of L-methionine were injected 15 minutes before the radioactive histidine, and the CO$_2$ collections were made. Formiminoglutaric acid was detected on the seventh day in the urine of the rat fed the vitamin B$_{12}$-deficient diet. On the 10th and 11th days the initial sequence of injections was repeated. The results are shown in Table II. In the normal rat, C$^{14}$O$_2$ excretion is slightly, but consistently, depressed by methionine. After a substantial
block in radioactive CO2 production occurs in the deficient rat, methionine has a marked stimulatory effect.

Formation of C14O2 from Sodium Formate-C14 and L-Serine-β-C14 in a Folic Acid- and Vitamin B12-Deficient Rat—Formate is activated by adenosine triphosphate and transferred to tetrahydrofolic acid (18). Plaut et al. (19) and Weinhouse and Friedman (20) have found that C14O2 production from radioactive formate is depressed in folic acid-deficient rats. Rabino

wits and Tabor (21) have shown that formate accumulates in the urine of these deficient animals. When small amounts of formate C14 (0.0 μC, 0.3 μmol) were injected into a doubly deficient and into a control rat, there was no difference in the formation of C14O2 (45% of the injected C14 was excreted). However, when 50 mg of nonradioactive sodium formate was added to the tracer dose, in a 4-hour period the control rat excreted 35% of the injected dose as C14O2, whereas a doubly deficient rat excreted 22%. “Preinjection” with methionine did not alter the amount of radioactive CO2 formed by either animal. In a sim

ilar experiment with 25 mg of labeled L-serine (containing 1.2 μC of C14) the doubly deficient rat excreted 20% of the labeled carbon as C14O2 compared to the control value of 30%. Again, “preinjection” with methionine did not affect the amount of C14O2.

Influence of Other Compounds on Formation of C14O2 from L-Histidine-2-C14 in Deficient Rats—Of the compounds tested (Table III) only methionine, betaine, methylmethionine sulfoxonium (S-methylmethionine), and ethionine were effective in stimulating C14O2 production by deficient animals. Homocysteine did not alter radioactive CO2 formation but did reduce urinary formiminogluta
tamic acid (see Fig. 1).

Fractionation of Urinary Metabolites of L-Histidine—A fractionation of metabolites of L-histidine-2-C14 occurring in the urines of control, folic acid-, vitamin B12-, and doubly deficient rats was carried out. Urines obtained from the same rats, after the addition of 1% L-methionine to the above deficient diets, were also analyzed. The following radioactive metabolites were measured quantitatively: imidazole-4(5)-acetic acid, L-ribosylimidazolacetic acid, urocanic acid, hydantoin propionic acid, formiminogluta
tamic acid, and formylisoglutamine, and an unknown compound (cf. Figs. 1 and 2 (17)). Except for formiminoglutamic acid, the amounts of radioactivity in the fractions of “deficient urine” corresponding to the individual metabolites did not differ significantly from those of the “control urine.” There is no evidence that dietary methionine influences any of the catabolic reactions of histidine, other than those involved in formiminoglutam
tic metabolism.

Table III

| Deficiency | Compound | Per cent of injected dose in C14O2 | Ability to reduce urinary formiminogluta
tamic acid excretion |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin B12</td>
<td>Control</td>
<td>2.9</td>
<td>Marked</td>
</tr>
<tr>
<td></td>
<td>L-Methionine</td>
<td>12.8</td>
<td>Moderate</td>
</tr>
<tr>
<td></td>
<td>Betaine hydrochloride</td>
<td>12.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sarcosine</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L-Methionine + sodium formate</td>
<td>11.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>1.8</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>L-Serine</td>
<td>1.5</td>
<td>None</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>Control</td>
<td>1.9</td>
<td>Marked</td>
</tr>
<tr>
<td></td>
<td>L-Methionine</td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Methionine sulphonic chloride</td>
<td>7.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Choline chloride</td>
<td>1.5</td>
<td>None</td>
</tr>
</tbody>
</table>
| | Dimethyl aceto
tetin | 2.5 | |
| | L-Methionine + L-serine | 8.9 | Moderate |
| | L-Ethionine | 11.4 | Moderate |
| Vitamin B12 and folic acid | Control | 1.4 | Marked |
| | L-Methionine | 3.7 | |
| | L-Homocysteine | 1.2 | Moderate |
| | L-Methionine + L-serine | 5.1 | |

a On successive days rats were treated by injection with L-
histidine-2-C14, then with methionine and L-histidine-2-C14, then with the other listed compounds and L-histidine-2-C14.

b The two vitamin B12-deficient animals were excreting approximately the same amounts of formiminoglutamate (about 40 μmoles per day) and weighed about 200 g. The doubly deficient rat weighed 350 g and was excreting in the order of 70 μmoles per day.

c Except for choline, dosages of 25 mg were administered intraperitoneally 15 minutes before the injection of histidine. Only 10 mg of choline chloride were used because of the toxicity of a larger amount.

d Animals were treated by injection with 1 μC (0.28 μmole) of L-histidine-2-C14.

Discussion

The mechanism by which methionine influences histidine metabolism in the rat is obscure. However, our results show that dietary methionine causes a significant increase in the release of “C-1 units” derived from histidine and that these “C-1 units” are released via the uric acid pathway. Other metabolic systems in which homocysteine and methionine influence the utilization of “C-1 units” have previously been described, but the activating mechanism of the amino acids in these systems is unclear. Berg (22) has shown a stimulatory effect of homocysteine on the formation of serine and purines from formate in unfractionated liver preparations. Nakoa and Greenberg (23) and Stevens and Sakami (24) have observed that methionine has an activating effect on the synthesis of methionine from formalde
yde and homocysteine in liver preparations. Bergmann et al. (25) have demonstrated that methionine and ethionine can reduce the accumulation of 4-aminomidazole-5-carboxamidocarboxymamide occurring in sulfonamide-inhibited cultures of Escherichia coli; minute amounts of p-aminobenzoic acid markedly stimulate this methionine effect. McRorie et al. (26) have shown that S-methylmethionine is even more effective than methionine in reversing the toxicity of sulphanilamide for E. coli.

A primary question raised by the “methionine effect” observed in the deficient rats (decreased urinary formiminoglutaric acid and increased C14O2 from L-histidine-2-C14) concerns the role of folic acid and vitamin B12. Does methionine permit a more efficient utilization of the available stores of folic acid and vitamin B12, or does a bypass mechanism exist which, in the presence of methionine, does not require these B vitamins? Data obtained with the intact animals do not permit any direct answer to this question. It can, however, be said that all of the obser-
vations are consistent with the view that methionine exerts its effect at the formiminoglutaric acid level. This suggests two interesting possibilities for investigation: one, that methionine, or a derivative, functions as an acceptor of the formimino group yielding a "high energy" sulfonium analogue which then can be further metabolized; and the other, that methionine permits a bypass of the B vitamins in the release of carbon 2 from histidine. Final proof of these speculations awaits studies in vivo.

The increases in CO$_2$ production from carbon 2 of histidine observed with compounds other than methionine can be accounted for by their ability to act as precursors of methionine. Stekol (27) has shown that the methyl groups of betaine are excellent sources of the methyl group of methionine (about 10 to 20 times more efficient than choline). Furthermore, S-methylmethionine is converted to methionine (28). The ability of ethionine to mimic methionine in this reaction is of interest in view of the fact that it too can be activated and can enter into transsulfhydration reactions (29-31). However, the manner in which ethionine competes with methionine and the scope of the reactions involved are poorly understood at the present time.

Injected homocysteine did not increase CO$_2$ production, but did decrease formiminoglutamic acid excretion. A possible explanation for this apparent discrepancy is that after the injection of homocysteine, the C-2 of histidine is used preferentially for the conversion of the homocysteine to methionine, for this conversion would occur slowly in the deficient animal, the amount of newly formed methionine in the tissues at any given time may not be great enough to stimulate significantly the production of CO$_2$ from C-2 of histidine. Thus, formiminoglutamate would be "utilized" and only give rise to C$_4$O$_2$ upon the oxidation of methionine-methyl to CO$_2$. Since the labeled methionine would be diluted with a large amount of unlabeled methionine in vivo, little C$_4$O$_2$ would be produced in the 4-hour collection period.

Attempts to implicate methionine in other folic acid requiring reactions of doubly deficient animals were without success. The C$_4$O$_2$ formed from L-serine-$eta$-C$^{14}$ or formate-C$^{14}$ is not increased by "reinjection" of the rats with methionine.

**SUMMARY**

It is concluded that methionine influences histidine metabolism by increasing the utilization of formiminoglutamic acid in folinic acid and vitamin B$_6$-deficient rats. In such animals, when methionine is given, there is an increased incorporation of label from L-histidine-$2$-C$^{14}$ into expired CO$_2$. Of a variety of compounds tested, only methionine, betaine, methylmethionine sulfoxide, and ethionine increased the production of C$_4$O$_2$ from L-histidine-$2$-C$^{14}$. The administration of methionine to the deficient rats also caused a reduction of the urinary excretion of formiminoglutamic acid. Homocysteine administration reduced the urinary formiminoglutamic acid but did not alter the respiratory C$_4$O$_2$. This stimulation, by methionine, of CO$_2$ formation from histidine was not depressed by the presence of other "1 carbon donors" (serine, glycine, or formate). In normal animals, the administration of methionine did not stimulate respiratory CO$_2$ production, but instead, consistently depressed it to a slight extent.

No differences were found in the distribution of metabolites of histidine-2-C$^{14}$ in the urines of folic acid-, vitamin B$_6$-, and doubly deficient rats.

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