FORMATE OXIDATION AND ITS INCORPORATION INTO URIC ACID IN FOLIC ACID DEFICIENCY

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Folic acid-deficient patients show evidence suggesting that intramedullary hemolysis is the primary mechanism of their anemia (*1,2*). If such intramedullary hemolysis involves total cellular destruction, one would anticipate increased nucleic acid synthesis as well as destruction. Indeed, on the basis of certain biochemical data Herbert *et al* have suggested that this may be the case (*3*). On the other hand, diminution in available folic acid would be expected to decrease the availability of monocarbon fragments needed for *de novo* purine and thymidine synthesis. There thus appears to be a conceptual dilemma in folic acid deficiency in which morphological and certain biochemical findings suggest that nucleic acid base synthesis should be increased while the presumed diminution in the availability of reduced folic acid suggests that purine synthesis should be decreased. We felt that a study of the fate of a cohort of purine molecules would aid in our understanding of this problem.

Cohorts of purines synthesized *in vivo* can be labeled by use of ^{14}C-monocarbon fragments arising from such sources as the #3 carbon atom of serine, the imidazole #2 carbon atom of histidine or the carbon atom of formate. The former two monocarbon fragment precursors have the liability of being primarily involved in protein synthesis and only a small fraction of their respective amino acid pools are metabolized through the monocarbon pool. This results in only a small fraction of ^{14}C label from the precursor material being incorporated into the purine cohort. An additional liability results from considerable recycling of the label from the protein back into the amino acid pool. This results in labeling of purines synthesized subsequent to the initial cohort. Formate, on the other hand, appears to be primarily metabolized by passage through the monocarbon pool (*4,5*) and, when folic acid reductase is inhibited, there is a marked diminution in its incorporation into purines and thymidine (*6,7*). These findings suggested that oxidation of formate to CO_2 and its incorporation into uric acid would be a measure of purine and monocarbon pool kinetics in folic acid deficiency. In the present study less than 5 μCi of high-specific-activity ^{14}C-formate was administered to normal and folic acid-deficient subjects prior to, and subsequent to, therapy with folic acid, and the appearance of $^{14}CO_2$ in the breath and ^{14}C-labeled uric acid in the urine was measured.

MATERIALS AND METHODS

^{14}C-formic acid was purchased from Calbiochem, Los Angeles, Calif. (S.A. 7.6 mCi/m*M*). The ^{14}C-sodium bicarbonate was obtained from New England Nuclear Corp., Boston, Mass. (S.A. 4.56 mCi/m*M*). The 4.64 μCi of ^{14}C-formic acid contained in 1 ml of saline solution or 2.29 μCi $H^{14}CO_3^-$ in 1 ml of saline adjusted to pH 8.0 by addition of NaOH was administered intravenously to the subject at the beginning of the study. The total rate of CO_2 exhalation and the appearance of $^{14}CO_2$ in the breath was measured using devices and procedures described previously (*8*). Such measurements were obtained for a minimum of 110 min. The bicarbonate study was performed at least 11 days after the administration of ^{14}C-formate. The $^{14}CO_2$ excretion per minute was divided by the total CO_2 excretion rate during that minute and the result multiplied by the average CO_2 excretion rate during the course of the study to correct for variations in CO_2 expiration rate. The values thus obtained were fit by functions consisting of sums of exponential terms using an appropriate minimization program for the CDC 6600 computer (*9*).

In an attempt to estimate the fraction of administered formate which was rapidly oxidized to CO_2, the amount of $^{14}CO_2$ expired during the initial 120

Received Oct. 28, 1969; original accepted Jan. 15, 1970.

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TABLE 1. CLINICAL DATA ON NORMAL AND FOLIC ACID-DEFICIENT SUBJECTS PRIOR TO AND SUBSEQUENT TO THERAPY

Patient	Age	Sex	Weight (kg)	Date of study	HGB (g%)	HCT	RBC (millions/ mm^3)	MCV	Serum folate (ng/ml)	Serum B_{12} (pg/ml)	Diagnosis
JB	27	M	73	4–28–69	15.1	44	5.23	83	4.7	—	N*
HS	32	M	85	5–5–69	15.4	45	5.24	86	6.9	—	N*
JL	51	F	45.5	3–19–69	12.3	35	2.90	120	1.8	‡	FAD†
				5–7–69	14.1	41	3.86	106	‖	—	
GC	40	M	78.4	4–2–69	7.1	19	1.84	104	1.0	220	FAD†
				4–30–69	15.3	45	4.36	103	7.3 ‖	—	
JH	64	F	76.4	4–11–69	5.7	17.5	1.38	127	1.0	370	FAD†
				5–23–69	12.1	38	3.75	101	‖	—	
HM	50	M	55	3–14–69	8.3	25.5	1.89	135	1.5	434	FAD†

* N = Control.
† FAD = Folic acid deficiency. JL had a malabsorption syndrome. GC, JH and HM had chronic alcoholism and nutritional folic acid deficiency.
‡ A Schilling test was performed 2 months prior to the first study and showed 10.5% absorption (normal 15–40%).
‖ JL received 10 mg folic acid p.o. for 6 weeks; GC received 20 mg folic acid p.o. for 1 week; and JH received 15 mg of folic acid for 4½ weeks prior to the repeat study.

min following injection of labeled formate (calculated from the exponential fitting function) was divided by that obtained following administration of labeled bicarbonate. The fraction of administered formate "fixed" or not directly oxidized to CO_2 was taken as 1 minus the fraction "directly" oxidized. These calculations obviously provide only rough estimates of these quantities. Following the formate injection, 24-hr urine samples for analysis of urinary uric acid were collected in bottles containing 10 ml chloroform. Urine collections were performed during the initial 11-day period. The samples were frozen within 12 hr after completion of collection and were kept frozen until processed. The urinary uric acid concentration was determined using a standard colorimetric assay (*10*). An aliquot of about 800 ml of each 24-hr urine sample was analyzed. To each sample 800 mg of uric acid in 80 ml of a 0.73% Li_2CO_3 solution (J. T. Baker Co.) were added as carrier and the sample evaporated under vacuum to a final volume of 200 ml. The urinary uric acid was then isolated and purified according to a method described by Sorensen (*11*). The 150–200 mg of the uric acid crystals thus obtained were suspended with Cabosil® (Packard Instrument Co., La Grange, Ill.) in scintillation fluid (0.05 gm POPOP, 4 gm PPO and 1 liter toluene). The samples were counted on a Nuclear-Chicago Mark I scintillation counter for ^{14}C. Each sample was internally standardized with a known amount of ^{14}C-toluene (New England Nuclear Corp.) to correct for quenching. Uric acid specific activity and total daily excretion of ^{14}C-uric acid were calculated in a routine fashion.

The clinical findings on the subjects of the present study are summarized on Table 1. Two normal subjects (JB and HS) served as controls. Both were males, and both were clinically healthy. They continued their normal but uncontrolled diet and activities while collecting urine. To exclude an inadvertent inclusion of a "normal" with hyperuricemia, serum uric acid concentration was determined on both subjects (JB 4.8 mg% and HS 6.3 mg%).

JL was referred to Donner Clinic with a malabsorption syndrome. Several years previously she had a subtotal gastrectomy for chronic gastric ulceration. Previously treated for iron deficiency, she slowly developed macrocytic red cell indices with decreasing hemoglobin concentration. The bone marrow aspirate showed megaloblastic changes. Following the initial study she was placed on 10 mg folic acid per day which was maintained for 6 weeks prior to performance of the repeat study.

GC, a 40-year-old male, had chronic alcoholism, macrocytic RBC and megaloblastic bone marrow changes first diagnosed as due to folic acid deficiency in the fall of 1968. At that time the patient responded to 100 μg folic acid p.o.q.d. and a normal hospital diet. He was readmitted with macrocytic anemia in the spring of 1969 at which time the initial formic acid studies were performed. Upon completion of the study the patient received 20 mg folic acid/day for 1 week and was advised to maintain an adequate

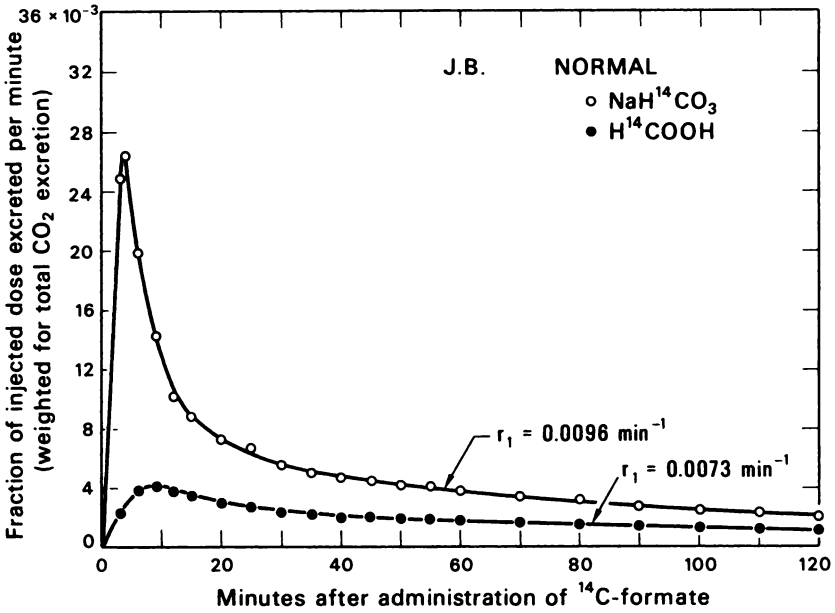

FIG. 1. Rate of $^{14}CO_2$ appearance in breath following i.v. administration of ^{14}C-bicarbonate and formate to normal subject JB. Ordinate is expressed as fraction of injected dose excreted/min ($\times 10^{-3}$) and abscissa is expressed as time in minutes following injection of labeled material. Best-fit function consisting of three exponential terms is shown for each curve.

diet. The formic acid incorporation study was repeated 28 days after the initial study.

JH, a 64-year-old female, had chronic alcoholism and macrocytic anemia with megaloblastic bone marrow changes. Subsequent to the initial study the patient received 15 mg folic acid q.d. for 4½ weeks prior to the repeat study.

HM, a 50-year-old male, had chronic alcoholism and macrocytic anemia with megaloblastic bone marrow changes.

All patients were placed on a folate free but otherwise normal hospital diet. Renal dysfunction was not observed in any of the patients (*11*).

Serum folic acid was assayed by the method described by Waters and Mollin (*12*) (normal range of 5–20 ng/ml). The B_{12} was determined by Clinical Laboratory Affiliates, Berkeley, using the radioisotope dilution technique and coated charcoal (*13*) (normal range 100–800 pg/ml).

RESULTS

Figure 1 graphically shows the rate of appearance of $^{14}CO_2$ in the breath subsequent to the administration of labeled bicarbonate (upper curve) and formate (lower curve) to normal subject JB. Subsequent to the administration of bicarbonate, there is a rapid initial excretion of $^{14}CO_2$, in this case reaching a maximum at 3 min, followed by a rapid diminution in the $^{14}CO_2$ expiration rate. In this case, the slope of the final exponential terms of

TABLE 2. VALUES FOR PARAMETERS OF FUNCTION* OBTAINED AS LEAST-SQUARES BEST FIT TO $^{14}CO_2$ EXPIRATION DATA

Patient	Date	^{14}C-formate A	B	C	r_1	r_2	r_3	^{14}C-HCO_3† r_1
JB	4–28–69	0.00271	0.00383	−0.0065	0.0073	0.0965	0.597	0.0096
HS	5–5–69	0.00372	0.182	−0.185	0.0128	0.4690	0.475	0.0143
JL	3–19–69	0.00434	0.169	−0.173	0.0125	0.5030	0.517	0.0165
	5–7–69	0.00293	0.00197	−0.0049	0.0100	0.0913	0.585	
GC	4–2–69	0.00085	0.00436	−0.0052	0.0031	0.0250	14.8	0.0115
	4–30–69	0.0023	0.00405	−0.0066	0.0097	0.1880	0.699	
JH	4–11–69	0.00220	0.156	−0.158	0.0042	0.379	0.379	0.0148
	5–23–69	0.00370	0.241	−0.244	0.0079	0.932	0.940	
HM	3–14–69	0.00421	0.155	−0.159	0.0120	0.411	0.421	0.0142

* $Ae^{-r_1 t} + Be^{-r_2 t} + Ce^{-r_3 t}$.
† Only the value of the final slope r_1, presented for the HCO_3^- data.

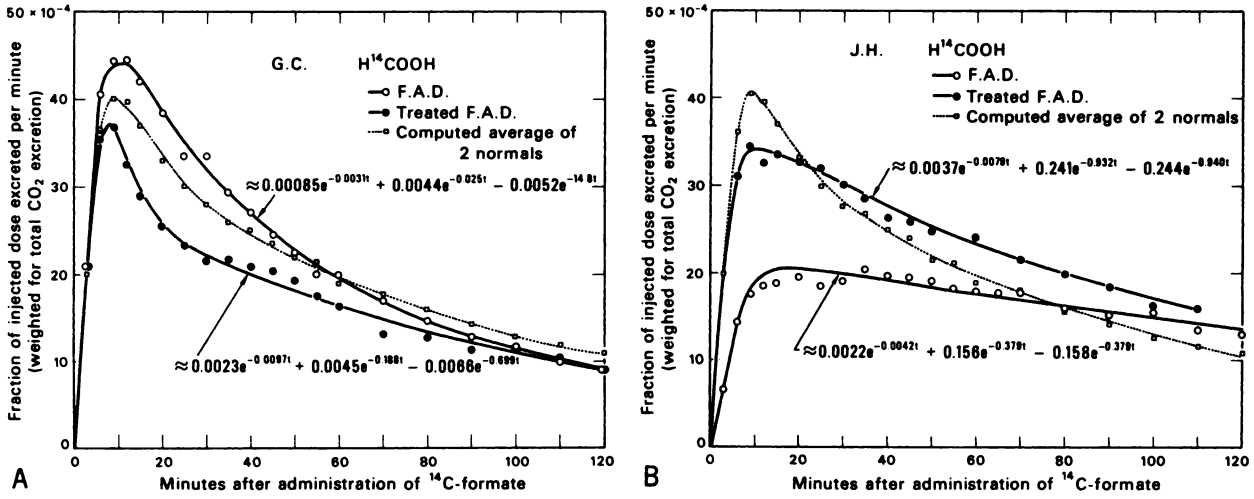

FIG. 2. Rate of $^{14}CO_2$ appearance in breath following i.v. administration of ^{14}C-formate to folic acid-deficient patients GC (A) and JH (B) prior to and subsequent to folic acid therapy. Ordinate is expressed as fraction of injected dose excreted/min ($\times 10^{14}$) and abscissa is expressed as time in minutes following injection of labeled material. Best-fit function consisting of three exponential terms is shown for each curve. Average $^{14}CO_2$ excretion obtained in two normal subjects (HS and JB) is shown for comparison.

the $^{14}CO_2$ curve obtained following administration of labeled bicarbonate was 0.0096 min^{-1}. Subsequent to the administration of labeled formate, the $^{14}CO_2$ expiration curve reaches a maximum value at 9–10 min with a subsequent slower diminution in the expiration rate than that seen following the administration of bicarbonate. The slope of the final exponential term in this case was 0.0073 min^{-1}.

Table 2 lists the values for the parameters determined by digital-computer least-squares best fit of a three exponential function to the $^{14}CO_2$ expiration data ($Ae^{-r_1t} + Be^{-r_2t} + Ce^{-r_3t}$) following administration of labeled formate. On the right-hand side of this table is listed the final slope of the $^{14}CO_2$ expiration curve following administration of labeled HCO_3^- (r_1).

TABLE 3. EXCRETION OF $^{14}CO_2$ IN BREATH AND ^{14}C-URIC ACID IN URINE FOLLOWING ADMINISTRATION OF ^{14}C-FORMATE TO NORMAL AND FOLIC ACID-DEFICIENT SUBJECTS PRIOR TO AND SUBSEQUENT TO THERAPY

Patient	24-hr urine uric acid excretion ± s.d. (mg)	A % ^{14}C from ^{14}C-formate excreted in breath during initial 2 hr	B % of ^{14}C from $H^{14}CO_3$ excreted in breath during initial 2 hr	C Estimated fraction of formate oxidized directly to $^{14}CO_2$(A/B)	D % ^{14}C from ^{14}C-formate excreted as urine uric acid in 11 days	E % of ^{14}C from "fixed" formate excreted as urine uric acid in 11 days D/1-C
JB	779 ± 225	26.8%	63.5	0.421	0.61	1.03
HS	890 ± 149	24.6%	82.9	0.297	1.14	1.62
JL	500 ± 131	27.1%	77.6	0.349	1.19	1.82
	442 ± 180	23.7%		0.305	1.10	1.43
GC	790 ± 324	26.6%	77.1	0.344	3.33	4.95
	890 ± 282	21.4%		0.278	1.27	1.75
JH	475 ± 132	20.4%	75.2	0.271	1.24	1.70
	348 ± 90	28.6%		0.379	0.52	0.81
HM	390 ± 158	27.0%	75.2	0.359	1.54	2.40
FAD avg.	583	25.2	76.2	0.331	1.83	2.72
Normal avg.	834	25.7	73.2	0.359	0.87	1.33
FAD after Rx avg.	560	24.5	76.2	0.321	0.96	1.33

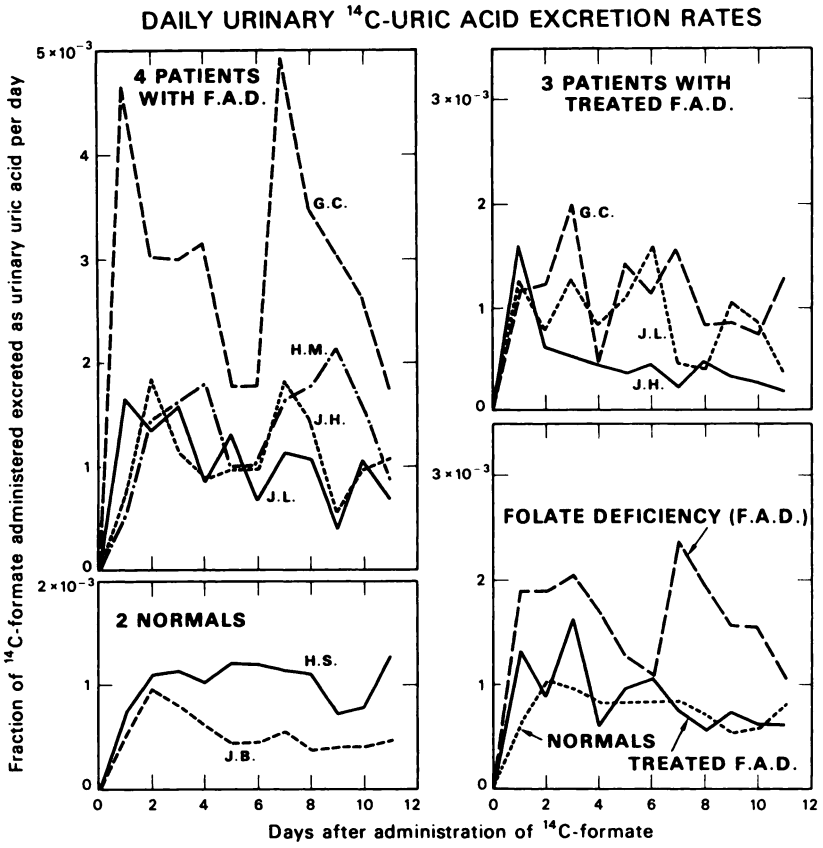

FIG. 3. Graphical presentation of the rate of urinary ^{14}C-uric acid excretion following administration of ^{14}C-formate to normal and folic acid-deficient subjects before and after treatment. Ordinate is expressed as fraction of administered ^{14}C-formate excreted as urinary ^{14}C-uric acid per 24-hr period. Abscissa represents time in days following i.v. administration ^{14}C-formate. Individual curves are shown for normal subjects (lower left), folic acid deficiency prior to therapy (upper left) and after therapy (upper right). Average curves for these three groups are shown in lower right portion of figure.

If formate is completely and rapidly oxidized to bicarbonate throughout the body, then the subsequent expiration rate of $^{14}CO_2$ in the breath would be a reflection of the turnover rate of the bicarbonate pool labeled following the oxidation of formate. However, the terminal slope of the formate curve in all cases studied was somewhat slower than that obtained following administration of labeled bicarbonate (cf. r_1 for formate and bicarbonate on Table 2).

The percent of ^{14}C from labeled bicarbonate excreted in the breath during the initial 2 hr is presented for each case on Table 3, and generally varied between 63 and 83%. The percent of ^{14}C from formate excreted as $^{14}CO_2$ in the breath during the initial 2 hr, also given on Table 3, varied between 20 and 28.6%.

Figure 2A and B graphically show the rate of excretion of $^{14}CO_2$ in the breath subsequent to the administration of ^{14}C-formate to patients with folic acid deficiency studied both before and after therapy with folic acid. Two curves are shown on this figure, each representing a different pattern of $^{14}CO_2$ excretion. A curve is also presented showing the average rate of excretion of $^{14}CO_2$ in the breath subsequent to the administration of ^{14}C-formate to two normal subjects. A greater rate of excretion of $^{14}CO_2$ was seen in patient GC (Fig. 2A) with folic acid deficiency prior to initiation of therapy than that seen both in the normal case and in the same patient subsequent to the administration of folic acid. Similar results were found in patient JL. Patient HM had a high excretion of $^{14}CO_2$ in the breath prior to therapy but was not studied subsequent to therapy. The rate of $^{14}CO_2$ excretion in the breath subsequent to the administration of ^{14}C-formate was markedly delayed and diminished in patient JH prior to therapy with folic acid (Fig. 2B). After 4½ weeks of folic acid administration (15 mg q.d.) to this patient, the rate of $^{14}CO_2$ excretion in the breath following administration of ^{14}C-formate approached the normal curve.

Figure 3 graphically presents the rate of ^{14}C excretion as uric acid in the urine subsequent to the administration of ^{14}C-formate. The ordinate is expressed as a fraction of the administered dose of ^{14}C-formate excreted as urinary uric acid/24-hr period. The rate of ^{14}C excretion as uric acid for individual cases are given for the normal subjects (lower left), patients with folic acid deficiency prior to therapy (upper left), and patients with folic acid deficiency subsequent to the administration of therapy (upper right). The averaged 24-hr excretion of ^{14}C-urinary uric acid for each of these three groups is presented in the lower right of this figure. From this figure it can be seen that prior to therapy there

was a greater excretion of ^{14}C as urinary uric acid in patients with folic acid deficiency as compared to normal subjects and that after treatment with folic acid the ^{14}C-uric acid excretion approximated the normal curve. Furthermore, prior to therapy the excretion of ^{14}C-uric acid in the urine showed two peaks, the first occurring between the second and third day and the second occurring at the seventh to eighth day subsequent to the administration of ^{14}C-formate. Although the second peak may also be present in the normal subjects and in the folic acid-deficient subjects subsequent to therapy, it is not as prominent as that seen in the folic acid-deficient patient prior to therapy.

For each subject in the present study, Table 3 lists: 24-hr urine acid excretion; percent ^{14}C from formate excreted in the breath during the initial 2-hr period; percent of ^{14}C from bicarbonate excreted in the breath during the initial 2-hr period; percent of ^{14}C from formate excreted as uric acid in the initial 11 days. In addition, the estimated fraction of formate directly oxidized to $^{14}CO_2$ is presented (calculated as $^{14}CO_2$ excretion following administration of formate divided by $^{14}CO_2$ excretion following administration of ^{14}C-bicarbonate). The percent of ^{14}C from "fixed" formate excreted as uric acid in the initial 11-day period is also presented (calculated by dividing the ^{14}C from formate excreted as uric acid in the initial 11-day period by the estimated amount of administered formate not directly oxidized to CO_2). Two cases (JL and GC) showed a decrease while a third (JH) showed an increase in $^{14}CO_2$ exhalation from formate following administration of folic acid therapy. In all cases the percent of ^{14}C from bicarbonate excreted in the breath in the initial 2-hr period was within normal limits, and the estimated fraction of formate oxidized to $^{14}CO_2$ for the various cases could not be individually distinguished from that seen in the normal subjects. In each case the percent of ^{14}C from formate excreted as uric acid during the initial 11 days was greater in patients with folic acid deficiency prior to therapy than that in the two normal subjects. Following therapy with folic acid the rate of ^{14}C from formate excreted as uric acid in the initial 11-day period approached the values obtained in the normal subjects.

DISCUSSION

Rapid attainment of the maximum rate of excretion of $^{14}CO_2$ in the breath subsequent to the administration of ^{14}C-formate in normal subjects and in patients with folic acid deficiency is consistent with the suggestion that the "formate" pool is rapidly turning over (*14*). The finding that the terminal slope of $^{14}CO_2$ excretion subsequent to the administration of formate is somewhat slower than that seen subsequent to the administration of bicarbonate further suggests that some of the ^{14}C label translocated from formate to other materials (e.g. the methyl group of methionine, choline, betaine, serine, glycine, etc.) is slowly oxidized to CO_2 and contributes to the rate of $^{14}CO_2$ excretion in the breath. The failure to show dramatic differences in $^{14}CO_2$ excretion following the administration of high-specific-activity ^{14}C-formate to patients with folic acid deficiency before and after treatment suggests that in these patients there is little impairment of formate metabolism when the exogenous formate "load" presented for metabolism is kept low. In previous studies with folic acid-deficient rats, loading doses of formate were administered (low-specific-activity ^{14}C-formate), and defects in metabolism of formate were noted (*4*). In similar folic acid-deficient subjects a marked diminution in the metabolism of the imidazole #2 carbon atom of histidine (*8*) and the #3 carbon atom of serine (*15*) has been demonstrated. Thus, the present results suggest that the metabolism of the imidazole #2 carbon atom of histidine or the #3 carbon atom of serine are much more affected by folic acid deficiency than formate.

The appearance of two distinct peaks in the rate of ^{14}C-uric acid excretion in the urine occurring at 2–3 days and 7–8 days in the patients with folic acid deficiency are quite similar to that previously noted by Krakoff and Balis (*16*) in the study of chronic granulocytic leukemia. These authors suggested that the second peak was related to the degradation of the nucleic acids contained within the proliferating leukemic cells. In that previous study the maximum rate of ^{14}C-uric acid excretion was 10–11 days while in the present study it is about 7–8 days. We propose that the present results may be due to intramedullary destruction of the cells with resultant degradation of their nucleic acid content. Digestion and absorption of sloughed intestinal mucosal cells may also contribute to their second peak. The finding that this peak occurs earlier in patients with folic acid deficiency than that seen with chronic granulocytic leukemia suggests an earlier destruction time for cells produced in folic acid deficiency than those produced in chronic granulocytic leukemia. The increased excretion of ^{14}C from formate in urinary uric acid found in the present studies suggests a paradoxical increase in purine synthesis in folic acid deficiency. However, if the formate and monocarbon fragment pool in folic

acid deficiency is smaller than that seen in normal subjects, a higher monocarbon fragment specific activity could be attained, resulting in abnormally large quantities of ^{14}C label in purines in the absence of an actual increase in overall purine synthesis. However, in rats it appears that that fraction of the formate pool which is unattached to tetrahydrofolic acid is actually increased in size in folic acid deficiency and excess formate is excreted in the urine (*5,14,17*). If the free formate pool is increased in size in human subjects with folic acid deficiency, then the increased ^{14}C excretion in urinary uric acid in the present studies may reflect an even greater increase in total purine synthesis in such subjects than that simply indicated by the ^{14}C values alone. Measurement of endogenous uric acid production in folic acid deficiency prior to, and subsequent to, therapy would be useful in answering this question. Unfortunately, in the present studies the purine content of the diet was not controlled and the average 24-hr purine excretion rates are relatively meaningless. Furthermore, no attempt was made to measure the loss of uric acid through the intestinal tract. Such measurements would be needed since there is some evidence that in megaloblastic anemia there is an increased excretion of uric acid in the intestinal tract (*18*).

CONCLUSIONS

To evaluate apparent discrepancies in monocarbon pool kinetics in folic acid deficiency (FAD), the metabolism of formate was studied in patients with FAD. Following i.v. administration of ^{14}C-formate, four patients with FAD excreted an average of 25.2% as $^{14}CO_2$ in the breath during the first 2 hr and 1.83% as ^{14}C-urinary uric acid during the first 11 days while two normal subjects excreted 25.7% in the breath as CO_2 and 0.87% as urinary uric acid and three of the FAD patients restudied after treatment with folic acid excreted 24.5% in the breath as CO_2 and 0.96% as urinary uric acid over the same time period. The apparent moderate increase in incorporation of ^{14}C from formate into early-appearing, labeled urinary uric acid suggests a normal or increased synthesis of rapidly catabolized purines in FAD. If these results simply reflect a diminished monocarbon fragment pool in FAD, then one must assume that the free formate pool is also diminished in size in patients with FAD and that in such patients exogenous formate rapidly attaches to tetrahydrofolate. The previously reported diminution in $^{14}CO_2$ production from the imidazole #2 carbon of histidine and the #3 carbon atom of serine in patients with folic acid deficiency contrasts with the relatively normal $^{14}CO_2$ production from formate in such patients in the present study. It thus appears that oxidation of the carbon atom of formate to CO_2 in human subjects is less affected by folic acid deficiency than the oxidation to CO_2 of the imidazole #2 atom of histidine or the #3 atom of serine. Following ^{14}C-formate administration to folic acid-deficient subjects the ^{14}C-urinary uric acid excretion shows two distinct peaks, one at 2–3 days and one at 7–8 days. The latter peak is not clearly seen either in normal subjects or in FAD after therapy, and may represent purine degradation secondary to intramedullary hemolysis or digestion and absorption of sloughed intestinal mucosal cells.

ACKNOWLEDGMENT

This work was supported under AEC contract No. W-7405-ENG-48. H. B. Stahelin is a trainee in nuclear medicine under NIH Training Grant No. 5-T01-GM 01752-02.

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