ALTERED CATABOLISM OF ^{14}C-FORMATE BY ERYTHROCYTES OF FOLIC ACID-DEFICIENT RATS: A POSSIBLE IN VITRO MEANS FOR DIFFERENTIAL DIAGNOSIS OF MEGALOBLASTIC ANEMIA IN MAN?

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A combined vibrating-reed electrometer-ionization chamber method appears to be a sensitive technique for studying biochemical processes in vivo in both animals (*1,2*) and man (*3*). This method has been reported recently as a nondestructive technique for differentiating between vitamin B_{12}-deficient and folic acid-deficient anemias in man in vivo with L-histidine(imidazole-2-^{14}C) (*4*) and sodium propionate-2-^{14}C (*5,6*). In the present study, we demonstrate that such a method is a useful tool for the same diagnostic purpose in vitro. The apparatus allows an instantaneous and continuous measurement of $^{14}CO_2$ production from ^{14}C-labeled L-histidine and formate by fresh mature red blood cells (RBC) of control, folic acid-deficient, and vitamin B_{12}-deficient rats in vitro.

METHODS AND MATERIALS

Preparation of experimental animals. Twenty-five rats (Canadian Breeding Laboratories, St-Constant, Co. Laprairie, Quebec) were divided into control, folic acid-deficient, and vitamin B_{12}-deficient groups. Folic acid-deficient and vitamin B_{12}-deficient diets were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. The folic acid-deficient diet had the following composition: gelatin, 8.0%; glucose, 45.8%; oil corn, 2.0%; oil and vegetable hydrogenate, 18.8%; salt mixture Briggs, 6.0%; vitamin-free casein, 20.0%; vitamin fortification with mixture free of folic acid. The vitamin B_{12}-deficient diet had the following composition: vitamin-free casein, 22.0%; sucrose, 68.5%; salt mixture No. 2 USP, 4.15%; vegetable oil (hydrogenated), 5.0%; iodinated casein, 0.05%; cysteine, 0.2%; choline, 0.1%; vitamin fortification mixture with exception of vitamin B_{12}. The experiments were conducted from 100 to 120 days after start of these diets (*7,8*). In these experiments, the control rats weighed an average of 180.46 gm at the beginning and 238.81 gm at the end of the experimental period. The folic acid-deficient and vitamin B_{12}-deficient rats weighed, respectively, an average of 176.7 and 161.95 gm at the beginning, and 176.63 and 270.20 gm at the end of the experimental period.

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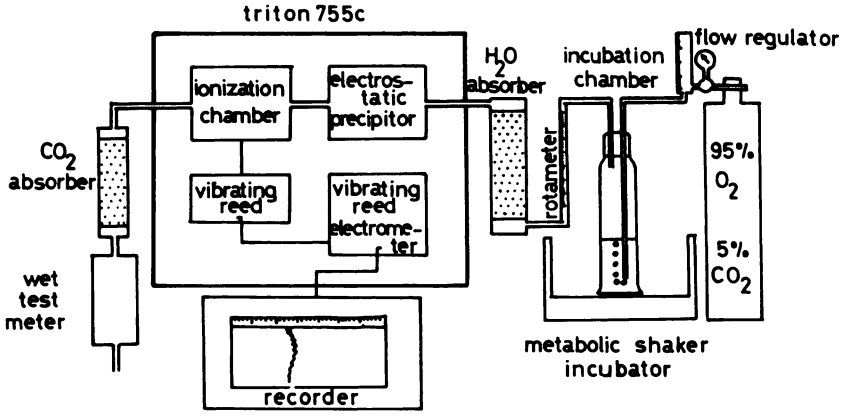

FIG. 1. Diagram of $^{14}CO_2$ analyzer.

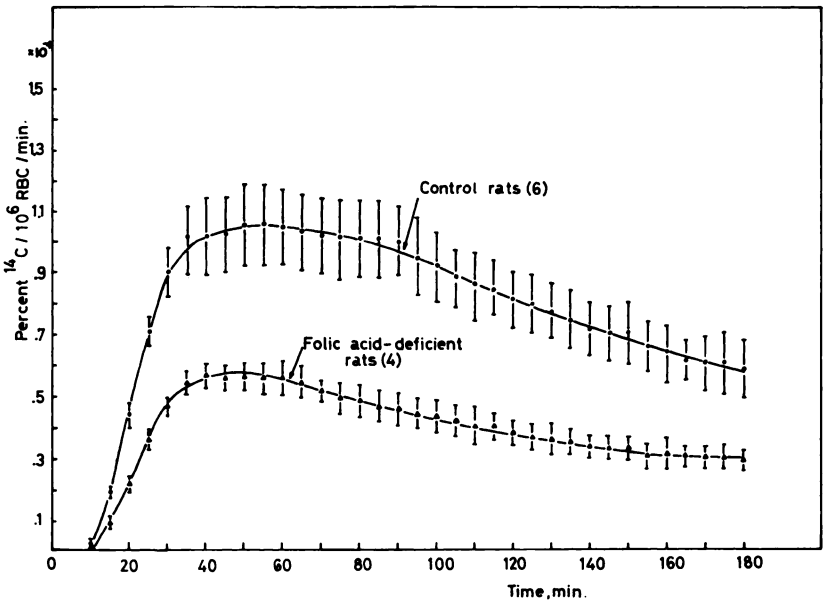

FIG. 2. Composite data of rate of $^{14}CO_2$ production from ^{14}C-formate by rat RBC of six control rats and four folic acid-deficient rats. Ordinate represents percent of ^{14}C excreted as $^{14}CO_2$ per million RBC per minute; abscissa represents time in minutes after incubation with ^{14}C-formate. Each point on curve represents mean of $^{14}CO_2$ excretion rate for each group of experiments, and length of 2 vertical bars through each point represents ±1 standard error of mean. Number of animals in each group is noted in parentheses.

$^{14}CO_2$ production studies. The apparatus devised for these studies is shown diagrammatically in Fig. 1.

For experimental procedures, rat red blood cells were washed twice in cold Ringer's solution (each 100 ml contains 860 mg NaCl, 30 mg KCl, and 33 mg $CaCl_2$, Baxter Laboratories of Canada Ltd., Malton, Ontario). Suspension of washed RBC in Gey's balanced salt solution (each 100 ml contains 700 mg NaCl, 37 mg KCl, 15 mg $Na_2HPO_4 \cdot 2H_2O$, 3 mg KH_2PO_4, 21 mg $MgCl_2 \cdot 6H_2O$, 17 mg $CaCl_2$, 100 mg glucose, and 227 mg $NaHCO_3$, Grand Island Biological Co., Grand Island, N.Y.) was then incubated at 37°C in the presence of 10 μCi of L-histidine (imidazole-2-^{14}C) SA: 22 mCi/m*M*, Amersham/Searle) or 2.5 μCi of ^{14}C-formate (SA: 52 mCi/m*M*, Amersham/Searle). Compressed gas with 95% O_2 and 5% CO_2 was passed continuously through the incubation chamber at a constant flow rate (100 cc/min) determined by a precision rotameter (Wright Co., London, England) and by a wet-test meter (Precision Scientific Co., Chicago, Ill.). The flow of gases then passes through a Triton-775 C (Johnston Laboratories, Inc., Cockeysville, Md.) which consists of a 1.2-liter ionization chamber and a vibrating-reed electrometer. Continuous graphical plotting of the $^{14}CO_2$ data was achieved by a chart recorder (Speedomax H, Leeds and Northrup Co., Philadelphia, Pa.). The background current from the device was determined when the flow gas passed through the incubation chamber containing RBC without ^{14}C-labeled materials. The ionization chamber was calibrated by introducing standard $^{14}CO_2$ gases into the ionization chamber and measuring current flow after equilibrium was reached. Standard $^{14}CO_2$ gases (Canadian Liquid Air, Sherbrooke, Quebec) were calibrated as follows. Ten liters of $^{14}CO_2$ gas were passed through 30 ml of absorber solution (1:2, v/v) solution of monoethanolamine (J. T. Baker Chemical Co., Phillipsburg, N.J.) in ethylene glycol monoethyl ether (Fisher Scientific Company, Fair Lane, N.J.) at a flow rate of 100 cc/min. Two milliliters of this absorber solution was added to 15 ml scintillation liquid of 1:2, v/v ethylene glycol monoethyl ether in toluene, containing 6 gm of 2,5-diphenyloxazole (PPO, scintillation grade) and 50 mg of 1,4-bis(2-(5-phenyloxazolyl))-benzene (POPOP, scintillation grade; Amersham/Searle).

The ^{14}C activity in the solution was determined by using a Packard Model 3385 liquid scintillation counter. The calibration factor of the ionization chamber was found to be 0.692×10^{-4} μCi/mV/min.

The apparatus was periodically tested for gas leaks to insure constancy of its performance.

RESULTS

Figure 2 represents composite data showing the rate of $^{14}CO_2$ production after incubation with ^{14}C-formate. It is clear that there is a qualitative difference between composite data of the normal $^{14}CO_2$ production curves and those obtained from folic acid-deficient rats. Figure 3 represents composite data showing the rate of $^{14}CO_2$ production by RBC of three control rats and five vitamin B_{12}-deficient rats after incubation with ^{14}C-formate. There is no qualitative difference between the composite data obtained from normal rats and those from vitamin B_{12}-deficient rats. Figure 4 represents composite

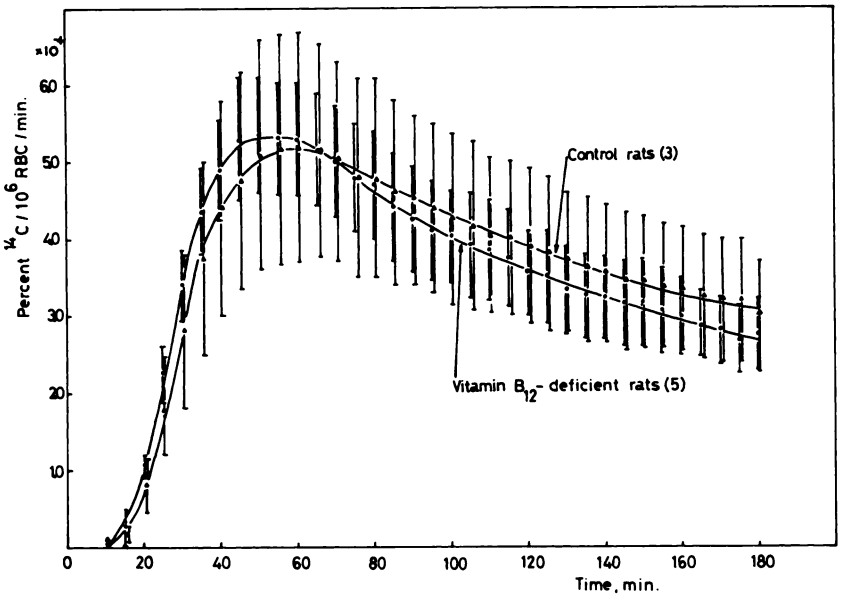

FIG. 3. Composite data of rate of $^{14}CO_2$ production from ^{14}C-formate by rat RBC of three control rats and five vitamin B_{12}-deficient rats. Ordinate represents percent of ^{14}C excreted as $^{14}CO_2$ per million RBC per minute; abscissa represents time in minutes after incubation with ^{14}C-formate. See Fig. 2 for details.

data of $^{14}CO_2$ production rates obtained from four control rats and four folic acid-deficient rats after incubation with L-histidine(imidazole-2-^{14}C). Similarly, there was no qualitative change between the composite data obtained from normal rats and those from folic acid-deficient rats.

For comparing various $^{14}CO_2$ curves, we determined the total fraction of the administered ^{14}C appearing as $^{14}CO_2$ by RBC within the first 180 min after incubation with a ^{14}C-labeled material. This parameter is a measure of the fraction of the labeled carbon atom of the substrate involved in oxidative processes leading to the production of $^{14}CO_2$. As shown in Table 1, there is significantly decreased $^{14}CO_2$ production from ^{14}C-formate during 180 min by RBC of folic acid deficient rats as compared to the normal ($p < 0.01$). The $^{14}CO_2$ production from this ^{14}C-labeled substrate, however, was unchanged in RBC of vitamin B_{12}-deficient rats ($p > 0.05$). Similarly, no significant change in CO_2 production from the #2 carbon atom of the imidazole ring of L-histidine was noted in RBC of folic acid-deficient rats ($p > 0.05$).

DISCUSSION

It is known that the ^{14}C from L-histidine(imidazole-2-^{14}C) and ^{14}C-formate are readily incorporated into the monocarbon pool attached to tetrahydrofolic acid (*4*). From this point, they may be either used in synthetic processes or rapidly oxidized to $^{14}CO_2$.

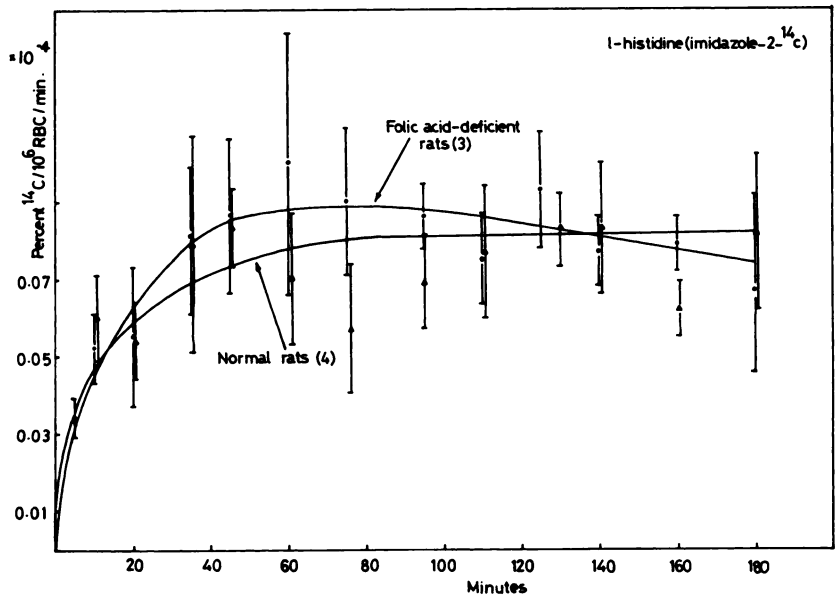

FIG. 4. Composite data of rate of $^{14}CO_2$ production from L-histidine (imidazole-2-^{14}C) by rat RBC of fourteen control rats and three folic acid-deficient rats. Ordinate represents percent of ^{14}C excreted as $^{14}CO_2$ per million RBC per minute; abscissa represents time in minutes after incubation with ^{14}C-histidine. See Fig. 2 for details.

In the present studies, we demonstrate that in control rats, approximately 4.1%/10^8 RBC and 0.16%/10^8 RBC of ^{14}C from the oxidation of the incubated ^{14}C-formate and L-histidine(imidazole-2-^{14}C), respectively, appear as $^{14}CO_2$ within the initial 180-min period. These data suggest that the enzymatic processes involved in the oxidation of the carbon atom from formate and of the #2 carbon atom of the imidazole ring from L-histidine by passage through the monocarbon pool must occur in normal and fresh mature RBC.

In RBC of folic acid-deficient rats there was a delay in the oxidation to $^{14}CO_2$ of ^{14}C-formate. This result is consistent with previous data which demonstrated a marked decrease in the excretion of $^{14}CO_2$ after the intravenous administration of ^{14}C-formate to folic acid-deficient rats in vivo (*7,8*). The $^{14}CO_2$ production from L-histidine(imidazole-2-^{14}C) by RBC of folic acid-deficient rats was, however, unchanged in our in vitro experiments. The results presented here are not in accordance with those obtained from folic acid-deficient humans (*4*), which demonstrated a decreased $^{14}CO_2$ appearance in the breath subsequent to the intravenous administration of ^{14}C-labeled histidine. The results obtained from our in vitro studies may therefore be explained by the fact that the oxidative catabolism of L-histidine did not occur largely in mature RBC. This suggests further that the major pathway in the oxidation from formate must proceed by passage through the monocarbon pool attached to tetrahydrofolic acid in mature RBC. The result of these studies confirm our previous finding which demonstrated a significant effect of ionizing radiation on the pattern of $^{14}CO_2$ production in the breath of irradiated rats after the intravenous injection of ^{14}C-formate. This is due to a radiation inactivation of tetrahydrofolic acid or the processes required for the production of this enzyme in irradiated rats (*9*).

It is noted that, in some experiments, no platelets and only approximately 4×10^2 leukocytes/million RBC were found in the cell suspension. Although the mass of these cellular elements are significantly less than that of the mass of RBC in the sample, their contribution to the overall metabolic activity of the sample may be considerable. The results obtained may reflect in part a variable content of leukocytes and platelets in rats with folic acid deficiency and vitamin B_{12} deficiency compared with normal rats. However, such variations cannot explain our results since these were relatively specific in that the difference was noted only in the metabolism of formate and not in the metabolism of L-histidine.

The fact that alterations occurred in the metabolism of formate in the RBC of folic acid-deficient rats but not in the RBC of vitamin B_{12}-deficient rats, suggests the possible application of this reliable vibrating-reed electrometer-ionization chamber method with ^{14}C-formate to the differential diagnosis of megaloblastic anemia in man.

TABLE 1. CUMULATIVE $^{14}CO_2$ PRODUCTION DURING INITIAL 180 MIN FROM ^{14}C-FORMATE AND L-HISTIDINE (IMIDAZOLE-2-^{14}C) BY MILLION RBC OF CONTROL, FOLIC ACID-DEFICIENT AND VITAMIN B_{12}-DEFICIENT RATS

Category	$^{14}CO_2$ excretion in 180 min (% $^{14}C/10^6$ RBC) ± s.e.
^{14}C-formate	
Control rats (6)	0.02047 ± 0.00225
Folic acid-deficient rats (4)	0.00820 ± 0.00083
Control rats (3)	0.06199 ± 0.01027
Vitamin B_{12}-deficient rats (5)	0.06342 ± 0.01766
L-histidine (imidazole-2-^{14}C)	
Control rats (4)	0.00161 ± 0.00020
Folic acid-deficient rats (3)	0.00200 ± 0.00057

* Number of animals in each group is noted in parenthesis.

CONCLUSIONS

A vibrating-reed electrometer-ionization chamber method has been used for instantaneous and continuous measurement of $^{14}CO_2$ production from ^{14}C-formate and L-histidine(imidazole-2-^{14}C) by RBC of control, folic acid-deficient, and vitamin B_{12}-deficient rats in vitro. A significantly decreased oxidation of ^{14}C-formate by RBC was noted in folic acid-deficient rats during 180 min compared with normal rats. The $^{14}CO_2$ production from this ^{14}C-labeled substrate, however, was unchanged in RBC of vitamin B_{12}-deficient rats. Similarly, no significant change in CO_2 production from the #2 carbon atom of the imidazole ring of L-histidine was noted in RBC of folic acid-deficient rats. The results of this study suggest that the major pathway of the oxidation from formate but not from the #2 carbon atom of the imidazole ring of L-histidine may proceed by passage through the monocarbon pool attached to tetrahydrofolate in mature RBC. Also this indicates a simple application of this reliable vibrating-reed electrometer-ionization chamber method to the differential diagnosis of megaloblastic anemias in man.

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