Effect of Carnitine and Ergothioneine on Human Platelet Metabolism[1] (34231)

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The present study was undertaken to look further into the role of the tricarboxylic acid (TCA) cycle in human platelets. Platelets derive most of their energy from glycolysis, producing large amounts of lactic acid in the process. Their energy metabolism is reviewed by Marcus and Zucker (1). An active pentose phosphate (PPh) cycle functions as the second most active pathway in the catabolism of glucose and is the chief source of carbon dioxide (CO_2) produced by platelets from glucose (2). Although platelets possess an operative TCA cycle, it is not a major source of energy. Yet 15–17% of the dry weight of platelets is lipid (1) and 25–30% of total lipid synthesis per unit of whole blood is attributable to platelets (3). With so much lipid to be synthesized, production of acetyl CoA from pyruvate oxidized via the TCA cycle must be important. If so, increased pyruvate oxidation should be demonstrable under conditions which stimulate extramitochondrial fatty acid synthesis. In other tissues such stimulation occurs when carnitine is added along with those substrates (acetate, glucose, pyruvate) which give rise to acetyl CoA within the mitochondria (4). Carnitine is known to act as a carrier of activated acyl groups across mitochondrial membranes (5). If this is important in platelet metabolism, carnitine should accelerate pyruvate oxidation.

Pyruvate-2-^{14}C was used for demonstrating increased TCA cycle activity in the *in vitro* system of the present studies. Addition of carnitine did increase TCA cycle activity, as judged by increased pyruvate oxidation, but with no concomitant increase in oxygen uptake.

Materials and Methods. Venous blood from human subjects, anticoagulated with ethylenediaminetetraacetic acid (EDTA), was processed as in earlier studies (2) to yield intact washed platelets by differential centrifugation in a siliconed system at 5°. Mammalian Krebs–Ringer–phosphate calcium-free buffer, pH 7.4, with added 0.01 *M* glucose and 0.1 vol of 1% EDTA in 0.7% sodium chloride was used as the washing buffer, and the same buffer without EDTA for the final suspension. Erythrocyte and leukocyte contamination was quantitated and found as before (2).

Basic incubation mixtures were as stated in footnotes of the tables. All flasks were run in duplicate. In determining $^{14}CO_2$ production, the overall method was as before (2), using the modification by Bressler and Friedberg (6) of the method of Snyder and Godfrey. A Nuclear-Chicago Mark I liquid scintillation spectrometer was used in the present studies. Lactic acid assays were done as before (2) by enzymatic method. Source of reagents was as before (2). The use of DL-carnitine and L-ergothioneine was dictated by commercial availability (Cal Bioch).

Oxygen consumption studies employed a YSI model 53 biological oxygen monitor, recording oxygen uptake for 5-min intervals after each addition to the basic incubation mixture which was contained in a Lucite chamber kept at 37° by a bath-stirrer assembly. The oxygen probe was connected through the oxygen monitor to a Sargent recorder model SRL. Probes were tested prior to each use and membranes were renewed if the probe failed to meet standard specifications. The basic incubation mixture consisted of 3 ml

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TABLE I. Production of $^{14}CO_2$ from Various Substrates by Human Platelets during 1 hr of Incubation at 30°, with and without Carnitine or Ergothioneine Added.

Platelets[a]	$^{14}CO_2$ production (μmoles/g of protein/hr; ± 1 SD)		
	No addition	+ Carnitine	+ Ergothioneine
+ Pyruvate-2-^{14}C (25 expts.)	18.1 ± 7.6	24.9 ± 9.2 ($p < .01$)	24.3 ± 7.7 ($p < .01$)
+ Pyruvate-1-^{14}C (8)	5.2 ± 6.5	6.8 ± 6.0	11.2 ± 8.5
+ Acetate-1-^{14}C (7)	33.3 ± 7.0	38.7 ± 5.5	41.9 ± 8.1
+ β-OH butyrate-3-^{14}C (1)	89.1	141.2	131.0

[a] Basic incubation mixture in Krebs–Ringer–phosphate buffer, calcium-free, pH 7.4, containing 0.01 *M* unlabeled glucose, with added diphosphopyridine nucleotide (NAD), 12 μmoles, and triphosphopyridine nucleotide (NADP), 5 μmoles, per 1.5 ml of mixture. Additional ^{14}C-labeled substrates indicated above were added in final 1.3 m*M* concentration. Carnitine and ergothioneine were added in final 2.4 m*M* concentration. Each flask received 1–3 mg of platelet protein as 1 ml of buffer-washed platelet suspension, the protein content being constant for all flasks of each experiment.

(3–6 mg of protein) of platelets, washed and suspended in buffer as before and as indicated in Table III. All reagents were aerated with filtered room air before use. Volume corrections were made for each addition in final calculations. Each run was repeated, using a second aliquot of the same platelet suspension, and results were averaged.

In studying gluconeogenesis, 0.7-ml frozen aliquots of incubation mixtures were thawed and passed through a 7.5 × 0.2-cm^2 mixed-bed resin column, consisting of Dowex-50-X8 (100–200 mesh) in the H^+ form and Dowex-1-X8 (100–200 mesh) in the HCO_3^- form, using the method of Corredor *et al.* (7). The sample was eluted with 2 ml of water into glass screw-cap vials to each of which was added 18 ml of a mixture containing 6 parts of Triton X-100 (Burris Products Co. Inc.) and 10 parts of PPO–POPOP (Packard Instrument Co., Inc.) in toluene.[2] Radioactivity of the vials was measured in a Packard model 3375 Tri-Carb liquid scintillation spectrometer. Corrections were made for quenching. In each experiment a control vial measured radioactivity of the eluate of a sample from an incubation mixture in which the reaction had been stopped at zero time. This "background" count was low and was deducted from each subsequent flask measured. Corredor *et al.* (7) determined by appropriate studies that of the intermediates and metabolites receiving the isotope label from substrates used, only neutral compounds could pass through the mixed-bed column. In three experiments in the present studies, using DL-lactate-2,3-^{14}C once and pyruvate-2-^{14}C, respectively, as substrates, eluates from mixed-bed resin columns were lyophilized, taken up in 0.2 ml of water and subjected to thin-layer chromatography (TLC) on MN 300G Cellulose (Macherey, Nagel and Co.), developing with *n*-butanol:pyridine:water (6:4:3) and spraying dried plates successively with silver nitrate (1 g in 1 ml of water + 30 ml of acetone), 1 *N* sodium hydroxide and (after 2 min) 1 *N* sodium thiosulfate to locate applied glucose, glycerophosphate, and glycerol standards. Bands were scraped from plates into vials containing 2 ml 0.5 *N* H_2SO_4 and counted after vigorous shaking and addition of 20 ml of Triton–toluene mixture as above.

Results. Table I shows the effect of DL-carnitine and L-ergothioneine on $^{14}CO_2$ production from ^{14}C-labeled pyruvate, acetate and β-OH butyrate. Production of $^{14}CO_2$ from all three substrates was enhanced, significantly so from pyruvate-2-^{14}C. The greater stability of pyruvate-2-^{14}C in part dictated its choice as a substrate in subsequent experiments. Ad-

[2] Four g of 2,5-diphenyloxazole (PPO) and 0.4 g of 1,4-bis-2-(5-phenyloxazolyle)-benzene (POPOP)/liter of toluene.

TABLE II. Production of Lactate by Human Platelets in 1 hr at 30°, with and without Added Carnitine, Ergothioneine, or Fluoroacetate.

Platelets[a] (8 expts.)	Lactate produced[b] (μmoles/g of protein/hr)
No addition	240 ± 41 (SD)
+ Carnitine, 4.8 m*M*	115 ± 55 ($p<0.01$)
+ Ergothioneine, 2.4 m*M*	156 ± 34 ($p<0.01$)
+ Fluoroacetate, 2.0 m*M*	359 ± 34 ($p<0.01$)

[a] In Krebs–Ringer–phosphate buffer, calcium-free, pH 7.4, containing 0.01 *M* unlabeled glucose and pyruvate, 1.3 m*M*, with added NAD, 12 μmoles, and NADP, 5 μmoles, per 1.5 ml of mixture.

[b] Assayed enzymatically; endogenous lactate (measured in each experiment in identical control flasks with reaction stopped at zero time by addition of acid) was deducted from the above values.

dition of 2.0 m*M* fluoroacetate as a TCA cycle inhibitor in 11 similar experiments revealed a mean of 94% inhibition of $^{14}CO_2$ production from pyruvate-2-^{14}C. The effect of addition of carnitine, ergothioneine, and fluoroacetate on lactate production was the reverse of that on $^{14}CO_2$ production in each instance, as shown in Table II. Gluconeogenesis was explored also as a further potential pathway in pyruvate metabolism, for reasons noted in discussion. No labeled glucose could be demonstrated from pyruvate-2-^{14}C, but it could be shown that neutral ^{14}C-labeled compound or compounds were produced, as shown in Table III. The effect of added carnitine, ergothioneine, and fluoroacetate on such production was the same as their effect on overall lactate production (Table II). Glucose, glycerol and urea are the only newly-produced neutral compounds known which could have been recovered from the incubation mixture used (Table III). Glucose was excluded by TLC, the label moving definitely in advance of glucose standards. The other compounds have not yet been definitely excluded.

No augmentation of oxygen consumption could be demonstrated upon addition of 2.2 m*M* carnitine or ergothioneine (Table IV). Expected inhibition was evident with 0.001 *M* cyanide; that with 0.1 *M* malonate was less than expected.

Substitution of addition of various 2.4 m*M* substances bearing some structural similarity to ergothioneine (choline, acetylcholine, betaine, 2-mercaptopurine) had no effect on $^{14}CO_2$ production from pyruvate 2-^{14}C, except for apparent inhibition in the presence of acetylcholine due to dilution of the acetate pool (Table V, A and B). However the sulfhydryl groups in ergothioneine may be important. Other SH compounds (cysteine and reduced glutathione) significantly increased $^{14}CO_2$ production from pyruvate-2-^{14}C (Table V, D and C). The striking inhibition of $^{14}CO_2$ production from pyruvate-2-^{14}C by a redox dye (DCI) was counteracted by the SH compound, ergothioneine, and not by carnitine (Table VI).

Discussion. The activity of the TCA cycle in human platelets is definitely increased in the presence of carnitine, as judged by production of $^{14}CO_2$ from pyruvate-2-^{14}C. This occurs without augmentation of oxygen uptake. Carnitine is known to undergo reversible esterification with activated acyl groups, to transport long-chain fatty acyl CoA compounds from site of formation outside to site of oxidation within mitochondria, and to

TABLE III. Production of ^{14}C-Labeled Neutral Compound from Pyruvate-2-^{14}C by Human Platelets in 1 hr at 30° with and without Carnitine, Ergothioneine, or Fluoroacetate Added.

Platelets[a]	Pyruvate (μmoles recovered as ^{14}C-neutral compound/g of protein)
+ Pyruvate-2-^{14}C (9 expts.)	17.3 ± 5.6 (SD)
+ carnitine, 4.8 m*M* (6)	7.1 ± 2.5 ($p<0.01$)
+ ergothioneine, 2.4 m*M* (6)	10.6 ± 3.1 ($p<0.01$)
+ fluoroacetate, 2.0 m*M* (7)	27.2 ± 8.7 ($p<0.01$)

[a] In Krebs–Ringer–phosphate buffer, calcium-free, pH 7.4, containing 0.01 *M* unlabeled glucose with added NAD, 12 μmoles, and NADP, 5 μmoles, per 1.5 ml of mixture. Labeled pyruvate-2-^{14}C was added in final 1.3 m*M* concentration. Other additions were as noted above.

TABLE IV. Oxygen Consumption by Human Platelets with and without Carnitine, Ergothioneine, Malonate, or Cyanide Added.

Platelets[a]	O_2 (μl/mg of protein/hr; 37°)
Baseline (16 expts.)	3.90 ± 1.02 (SD)
With added:	Mean % inhibition
DL-Carnitine, 2.4 m*M* (5)	2
L-Ergothioneine, 2.2 m*M* (6)	2
Malonate, 0.1 *M* (2)	14
Cyanide, 0.001 *M* (2)	73

[a] In aerated Krebs–Ringer–phosphate buffer, calcium-free, pH 7.4, with 0.01 *M* glucose and 0.004 *M* pyruvate.

TABLE V. Effect of Other Compounds Structurally Related to Ergothioneine on $^{14}CO_2$ Production from Pyruvate-2-^{14}C by Human Platelets during 1 hr of Incubation at 30°.

Platelets[a]	$^{14}CO_2$ production (μmoles/g of protein)
(A) + Pyruvate-2-^{14}C (6 expts.)	22.7 ± 8.9
+ acetylcholine	12.0 ± 5.3 ($p<0.05$)
(B) + Pyruvate-2-^{14}C (1)	31.5
+ carnitine	43.8
+ ergothioneine	42.0
+ methionine	28.8
+ choline	30.1
+ acetylcholine	13.7
+ betaine	30.6
+ 2-mercaptopurine	27.2
(C) + Pyruvate-2-^{14}C (av, 2 expts.)	8.1
+ carnitine	13.6
+ ergothioneine	14.5
+ cysteine	12.3
(D) + Pyruvate-2-^{14}C (2)	15.0
+ carnitine	22.2
+ ergothioneine	20.0
+ reduced glutathione	20.5

[a] In Krebs–Ringer–phosphate buffer, calcium-free, pH 7.4, containing 0.01 *M* unlabeled glucose, with added NAD, 12 μmoles, and NADP, 5 μmoles, per 1.5 ml of mixture. Pyruvate-2-^{14}C was added in final 1.3 m*M* concentration. Other substances listed above were added in final 2.4 m*M* concentration, except 2-mercaptopurine which was poorly soluble and added in partial suspension. All additives were brought to pH 7.4 before use.

transport acetyl CoA from its site of formation within to outside mitochondria for long-chain fatty acid synthesis (5). The evidences of increased intramitochondrial oxidation of pyruvate in the presence of carnitine, without an increase in oxygen uptake, suggest an increased utilization of acetyl CoA for transport outside mitochondria for long-chain fatty acid synthesis, rather than increased respiration to meet energy requirements of the platelets. This is consonant with present concepts of energy sources in platelets. The fact that less $^{14}CO_2$ was produced from pyruvate-1-^{14}C than from pyruvate-2-^{14}C may in part be due to randomization of the ^{14}C label from pyruvate-2-^{14}C at the succinate step during its initial full TCA cycle before release of $^{14}CO_2$ in its second cycle. It may also relate

TABLE VI. Counteraction by Ergothioneine of Effect of Redox Dye[a] on $^{14}CO_2$ Production from Pyruvate-2-^{14}C by Human Platelets during 1 hr of Incubation at 30°.

Platelets[b]	$^{14}CO_2$ production (μmoles/g of protein/hr; ± 1 SD)
+ Pyruvate-2-^{14}C (8 expts.)	14.1 ± 5.8
+ carnitine, 2.4 m*M*	20.6 ± 7.3
+ ergothioneine, 2.4 m*M*	20.6 ± 6.0
+ DCI, 0.18 m*M*	5.1 ± 3.2
+ DCI + carnitine	6.2 ± 3.6
+ DCI + ergothioneine	20.3 ± 7.1

[a] Dichlorphenolindophenol (DCI).

[b] In Krebs–Ringer–phosphate buffer, calcium-free, pH 7.4, containing 0.01 *M* unlabeled glucose with added NAD, 12 μmoles, and NADP, 5 μmoles, per 1.5 ml mixture. Labeled pyruvate-2-^{14}C was added in final 1.3 m*M* concentration.

to CO_2-fixing reactions involving malic acid dehydrogenase and pyruvic acid carboxylase, since some reversal of glycolysis is suggested by the finding of ^{14}C-labeled neutral compound in the reaction mixtures. The compound has not yet been identified, but it is not glucose. Glycerol and urea remain as potential neutral compounds not yet excluded but possible in the incubation mixtures used. The data indicate the possibility of some degree of reversal of glycolysis by human platelets as surmised by Karpatkin whose

data suggested conversion of citrate to lactate by platelets (8).

Ergothioneine was employed because like carnitine it is a biological quaternary amine widely present in mammalian tissues. However, unlike carnitine, it is a sulfhydryl compound and is without known biological function. It appears to be derived from dietary sources, but its dietary deprivation in animals has not been associated with apparent adverse effect. Melville (9) called attention to the avidity with which dietary ergothioneine is incorporated into mammalian tissues, the tenacity with which it is held there and the ubiquity of its distribution. He postulated an undefined metabolic role or perhaps one functional only under conditions of stress. Spicer *et al.* (10) postulated a physiologic role in maintaining hemoglobin in the ferrous form. The present study demonstrates its ability *in vitro* to increase oxidation of pyruvate in human platelets while decreasing production of lactate and of a neutral compound labeled from pyruvate-2-^{14}C which possibly may be an intermediate resulting from partial reversal of glycolysis. These effects are the same as those exhibited by carnitine. However it seems probable that its action differs from that of carnitine. Reproduction of the same effect on pyruvate oxidation by other sulfhydryl compounds suggests that the effect of ergothioneine may be related at least in part to its SH group, perhaps nonspecifically keeping CoA·SH reduced.

Summary. Addition of carnitine or ergothioneine to human platelets in an *in vitro* incubation system resulted in significantly increased production of $^{14}CO_2$ from pyruvate-2-^{14}C, and decreased production of lactate from glucose and of neutral compound from pyruvate. Oxygen consumption was unaffected. Results suggested that in the *in vitro* system employed, the importance of tricarboxylic acid cycle activity related more significantly to fatty acid metabolism than to respiration.

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