It is of interest that the mitotic response to injury is associated with a latent period of about 2 days. Whatever the signal is that denotes a stimulus for vessel cell proliferation, it evidently requires a time-consuming reaction to become effective. The present data suggest that the replacement cells are derived from the blood vessel itself, rather than from a remote pool of proliferating cells. It has been suggested that vascular endothelium may be derived from blood leukocytes(4,5). This hypothesis is not supported by the present observation that labeled cells failed to appear in injured vessels when tritiated thymidine was administered prior to injury, although labeled blood leukocytes were abundant.

Since different types of vascular injury are accompanied by proliferative activity, a practical implication of the present studies is that increased labeling of blood vessels may be a sensitive indicator of damage. Studies are in progress which will use the vessel labeling technique to determine the effects of endotoxin and of experimental thrombocytopenia purpura.

Summary. Rabbit blood vessel cells were labeled with tritiated thymidine. Labeled cells were seen in all layers of all vessels of normal, adult animals, although the incidence was low. Following chemical or physical injury, increased mitotic activity developed after a latent period of 2 days. Injection of isotope prior to vascular injury was unaccompanied by the appearance of labeled cells in the vessel wall.

1. Altschul, R., in Blood Platelets, Little, Brown & Company, Boston, Mass., 1961, p23-39.

2. Spraragen, S. C., Bond, V. P., Dahl, L. K., Circulation Res., 1962, v11, 329.

3. Leblond, C. P., Messier, B., Kopriwa, B., Lab. Invest., 1959, v8, 296.

4. Moschcowitz, E., Arch. Path., 1950, v49, p147.

5. Wylie, E. J., Kerr, E., Davies, O., Surg., Gynecol., & Obstet., 1951, v93, 257.

Received April 28, 1967. P.S.E.B.M., 1967, v125.

Carnitine Stimulated Transport of the Intermediates of Long Chain Fatty Acid β Oxidation in Liver and Heart Mitochondria.* (32313)

VINCENT W. HOLLIS, JR.,† AND MELVIN BLECHER
Department of Biochemistry, Schools of Medicine and Dentistry, Georgetown University, Washington, D. C.

We have recently reported the chemical synthesis(1) and mitochondrial oxidation(2) of three 1-^{14}C-labeled intermediates of the β oxidation of palmitate, namely, *trans*-2-hexadecenoate, 3-hydroxyhexadecanoate and 3-ketohexadecanoate. In the light of the postulates(3-5) that the carnitine-induced stimulation of the mitochondrial oxidation of long-chain *saturated* fatty acids(6,7) is mediated through the function of O-acyl carnitine as a carrier of saturated fatty acids across mitochondrial membranes, we undertook to examine the possible effects of carnitine on the oxidation of the aforementioned intermediates of palmitate β oxidation by liver and heart muscle mitochondria. Since Norun has reported recently(8) that the enzyme catalyzing the reversible formation of O-palmityl carnitine, namely, palmityl CoA-carnitine palmityl transferase, is elevated in activity in livers of alloxan-diabetic rats, we have also examined the effects of carnitine on the oxidation of the palmitate β oxidation intermediates by liver mitochondria from diabetic rats.

The data presented here demonstrate that carnitine stimulated oxidation not only of palmitate, but also of two of the three β oxidation derivatives of palmitate, by liver and heart muscle mitochondria from normal and diabetic rats. With liver mitochondria, carnitine also increased the accumulation of

* Supported by grant AM-06208 from Nat. Inst. Health, Bethesda, Md.

† Present address: National Inst. of Arthritis & Metab. Dis., Bethesda, Md.

acetoacetate from palmitate and β oxidation intermediates. Diabetes did not modify the carnitine effect.

A preliminary report of these studies has appeared(9).

Experimental procedures. [1-^{14}C] Palmitic acid was obtained from Nuclear Research Chemicals, Orlando, Florida, and [1-^{14}C] *trans*-2-hexadecenoic acid, [1-^{14}C] DL-3-hydroxy-hexadecanoic acid, [1-^{14}C] 3-ketohexadecanoic acid were synthesized as previously reported(1). Crystalline bovine serum albumin (BSA) was obtained from Pentex, Inc., DL-carnitine-HCl from Calbiochem, CoA from Pabst Labs. and ATP and β-NAD from Sigma Chem. Co.

Male rats, 7- to 8-weeks old, obtained from Holtzman Rat Co., were fed Purina laboratory chow, but were fasted overnight prior to use. Diabetes was induced by a single intraperitoneal injection of alloxan (20 mg/100 g body wt) following a 24-hour fast; the animals had free access to the stock diet thereafter. Only rats with blood glucose levels above 300 mg% and glucosuria were used as diabetic animals; this condition usually resulted within 4 days after administration of alloxan.

Liver and heart muscle tissues were homogenized in a solution composed of 0.01 M Tris-HCl buffer (pH 7.4)-0.001 M sodium EDTA-0.25 M sucrose; following removal of nuclei and cellular debris at 700 $\times$ *g*, liver and heart mitochondria were isolated at 10,000 $\times$ *g* and 20,000 $\times$ *g*, respectively, according to standard techniques.

Incubations were performed in 25 ml flasks fitted with serum caps and hanging glass center wells. The standard incubation medium for studies with liver mitochondria contained the following components in a total volume of 3 ml: 420 μmoles KCl, 0.3 μmole succinate, 9 μmoles $MgSO_4$, 6 μmoles ATP, 36 μmoles potassium phosphate buffer (pH 7.4), 250 μmoles sucrose, 1 μmole EDTA, 10 μmoles Tris-HCl buffer (pH 7.4), 3 μmoles of fatty acid substrate as the sodium salt, and, when used, 0.45 μmole BSA and 1.5 μmoles DL-carnitine-HCl. Each vessel contained 8 to 10 mg of mitochondrial protein, except with the 3-ketohexadecanoate substrate where only 0.25 mg of mitochondrial protein was required to obtain zero order kinetics.

The incubation medium for heart mitochondria contained, in addition to the above listed components, 7.5 μmoles β-NAD and 0.12 μmole CoA; fatty acid substrates were 0.3 μmole; where used, BSA was 0.045 μmole. The amount of mitochondrial protein used varied with each substrate, *i.e.,* 2 mg with palmitate, 0.5 to 0.7 mg with *trans*-2-hexadecenoate and DL-3-hydroxyhexadecanoate, and 0.02 mg with 3-ketohexadecanoate; in these ranges, zero order kinetics were obtained.

Following incubation for 1 hour at 37°, 0.3 ml of Hyamine hydroxide (Rohm and Haas; 1 M in Methanol) was injected through the serum cap into the center well, and 0.3 ml of citric acid (0.5 g per ml) into the incubation mixture. The flasks were then shaken for an additional 30 minutes to permit complete absorption of radioactive carbon dioxide by the Hyamine. Radioactive Hyamine carbonates were transferred to counting vials containing 10 ml of 0.4% 2,5-diphenyloxazole-0.05% 1,4-bis-2-(5-phenyloxazolyl) benzene in toluene for determination of radioactivity in a Nuclear-Chicago liquid scintillation spectrometer; quench corrections were made by the channels ratio method. In those studies in which acetoacetic acid was assayed, fresh center well assemblies were then placed in the incubation flasks, 0.3 ml of Hyamine was injected into the center wells, and 0.4 ml of an aniline-citrate reagent (0.5 g per ml each of aniline hydrochloride and citric acid in water) into the incubation mixtures; flasks were shaken for 60 minutes, during which time the carboxyl carbon atom of acetoacetic acid, quantitatively removed as carbon dioxide, was absorbed in the Hyamine.

The results are expressed in terms of mμmoles of substrate oxidized to $^{14}CO_2$ or converted to acetoacetate per mg of mitochondrial protein during one hour of incubation; values are corrected for $^{14}CO_2$ produced in the absence of mitochondria.

Results. The effects of carnitine on the oxidation of the 4 substrates to $^{14}CO_2$ by rat liver mitochondria are shown in Table I. It is ap-

TABLE I. Effects of Carnitine on Oxidation to $^{14}CO_2$ of Palmitate and Its β Oxidation Products by Liver Mitochondria from Normal Rats.

1-^{14}C substrate	mμmoles oxidized to $^{14}CO_2$ per mg protein per hr		
	Basal	Carnitine	% Increase
Palmitate	15.05 ± 3.29 *	24.47 ± 3.75	63
	(25.11 ± 6.66) †	(43.81 ± 5.72)	(43)
Trans-2-Hexadecenoate	9.68 ± .69	14.39 ± 1.62	49
	(20.29 ± 4.89)	(32.32 ± 3.84)	(59)
DL-3-Hydroxyhexadecanoate	17.84 ± .63	27.86 ± 3.22	56
	(27.62 ± .78)	(37.03 ± 2.78)	(70)
3-Ketohexadecanoate	2881 ± 243	3312 ± 318	—

* Mean ± S.E.$_M$ for 5 experiments.
† Values in parentheses were obtained in the presence of 0.15 mM BSA.

parent that carnitine significantly stimulated the oxidation of all substrates except 3-ketohexadecanoate. In the presence of BSA, which is frequently used to solubilize long chain fatty acids, basal oxidations were significantly elevated; however, BSA did not significantly alter the extent of the effect of carnitine. No results are reported for the mitochondrial oxidation of 3-ketohexadecanoate in the presence of BSA since this protein, either crystalline or fatty acid-poor, caused extensive, non-enzymatic decarboxylation of the 3-keto compound. Concurrent experiments on the effects of carnitine on the production of acetoacetate revealed a pattern similar to that seen when oxidation to carbon dioxide was the parameter measured, that is, an increase in the accumulation of acetoacetate from all these substrates (Table II); as in Table I, BSA increased basal values but, in addition, greatly magnified the effect of carnitine.

Similar studies were carried out with rat heart muscle mitochondria, since it has been reported that this tissue preferentially oxidizes fatty acids(10), and since the carnitine mechanism has been thoroughly demonstrated in this tissue(7). The results of these experiments are shown in Table III. It is apparent from these data that carnitine stimulated oxidations of palmitate and its unsaturated and 3-hydroxy derivatives; carnitine effects in this tissue were much greater than in liver mitochondria, a distinction previously reported for palmitate oxidation(11). However, as with liver, there was no significant effect of carnitine on the oxidation of 3-keto derivative by heart mitochondria. Table III also illustrates that BSA caused a 2-to-3-fold stimulation of the carnitine effect on fatty acid oxidation, an observation made previously with palmitate oxidation in the presence of BSA(11). We have also observed, as have other investigators (6), that acetoacetate did not accumulate in

TABLE II. Effects of Carnitine on Accumulation of Acetoacetate from Fatty Acids in Liver Mitochondria from Normal Rats.

1-^{14}C substrate	mμmoles of acetoacetate formed per mg protein per hr‡		
	Basal	Carnitine	% Increase
Palmitate	5.24 ± 1.22 *	10.86 ± 1.07	96
	(17.00 ± 6.65) †	(58.60 ±11.13)	(245)
Trans-2-Hexadecenoate	3.89 ± .51	6.33 ± .96	63
	(7.42 ± 2.04)	(35.05 ± 7.91)	(372)
DL-3-Hydroxyhexadecanoate	2.15 ± .36	4.86 ± .78	126
	(3.55 ± .26)	(34.27 ± 3.45)	(865)

* Mean ± S.E.$_M$ for 5 experiments.
† Values in parentheses were obtained in the presence of 0.15 mM BSA.
‡ Values were calculated on the assumption that the carbonyl and carboxyl (isolated) carbon atoms of acetoacetate contained equal amounts of radioactivity(12). The conversion of [1-^{14}C] 3-ketohexadecanoate to acetoacetate was not determined, since aniline citrate also decarboxylated the former.

TABLE III. Effects of Carnitine on Oxidation to $^{14}CO_2$ of Palmitate and Its β Oxidation Products by Heart Muscle Mitochondria.

1-^{14}C substrate	mμmoles oxidized to $^{14}CO_2$ per mg protein per hr		
	Basal	Carnitine	% Increase
Palmitate	4.49 ± .45 *	22.06 ± 4.01	391
	(4.72 ± .43) †	(43.70 ± 6.85)	(826)
Trans-2-Hexadecenoate	1.83 ± .25	4.05 ± .99	121
	(2.03 ± .54)	(13.82 ± 4.23)	(582)
DL-3-Hydroxyhexadecanoate	4.76 ± .72	6.99 ± 1.69	41
	(5.01 ±1.10)	(10.22 ± 1.87)	(104)
3-Ketohexadecanoate	3416 ± 297	3593 ± 278	—

* Mean ± S.E.$_M$ for 5 experiments.
† Values in parentheses were obtained in the presence of 0.015 mM bovine serum albumin.

TABLE IV. Effects of Carnitine on Oxidation of Palmitate and Its β Oxidation Intermediates by Liver Mitochondria from Diabetic Rats.

1-^{14}C substrate	mμmoles oxidized to $^{14}CO_2$ per mg protein per hr		
	Basal	Carnitine	% Increase
Palmitate	10.10 ± 1.79*	17.35 ± 3.61	72
Trans-2-Hexadecenoate	7.64 ± 2.48	10.93 ± 3.80	43
DL-3-Hydroxyhexadecanoate	9.97 ± 1.57	15.40 ± 2.38	54
3-Ketohexadecanoate	2722 ± 232	2873 ± 237	—

* Mean ± S.E.$_M$ for 5 experiments.

this tissue, and no effects of carnitine were seen on the formation of this product.

Since it has been reported that hepatic fatty acyl-carnitine transferase activity was elevated in diabetic rats(8), we examined the effects of carnitine on the oxidation of the 4 substrates by liver mitochondria from diabetic rats; the results are given in Table IV. As with normal tissue, carnitine stimulated the oxidation of palmitate, trans-2-hexadecenoate and DL-3-hydroxyhexadecanoate, but not 3-ketohexadecanoate. It should be noted that the carnitine effect with tissue from diabetic animals was no greater than that observed with normal liver mitochondria (compare Table I and IV). Furthermore, diabetes appeared to depress somewhat the basal oxidation of all substrates except the 3-ketoderivative; carnitine increased these values to the normal range.

The production of acetoacetate from all substrates by liver mitochondria from diabetic animals (Table V) was similar to that obtained with normal tissue (Table II); however, diabetes significantly increased the magnitude of the carnitine effect.

Discussion. In interpreting these results we were concerned, of course, with the physiological significance of these carnitine effects. What could be the function of a carnitine-stimulatable translocation mechanism for intermediates of the *oxidation* of long-chain fatty acids if these are produced exclusively by and remain *within* the mitochondrion? A clue to a possible function has been provided by the recent description by Nugteren(13) of a rat liver microsomal chain elongation mechanism involving malonyl CoA and NADPH, in which the aforementioned compounds, namely, non-protein bound 3-ketoacyl-CoA, 3-hydroxyacyl-CoA and trans-2-enoyl-CoA derivatives of palmitate occurred successively as intermediates. It may be postulated, therefore, that, when required, such fatty acid intermediates produced either by oxidative or reductive reactions, might be translocated across mitochondrial and microsomal membranes by the carnitine mechanism, for oxidative and/or reductive purposes.

In contrast to Norum's findings of increased hepatic fatty acyl carnitine transferase activity in diabetic rats(8) we were unable to demonstrate in these experiments any effect of carnitine on the oxidation of the 4 sub-

TABLE V. Effects of Carnitine on Production of Acetoacetate from Fatty Acids in Liver Mitochondria from Diabetic Rats.

1-^{14}C substrate	mμmoles of acetoacetate formed per mg protein per hr†		
	Basal	Carnitine	% Increase
Palmitate	2.82 ± .69*	11.80 ± 4.70	318
Trans-2-Hexadecenoate	2.63 ± .66	5.88 ± .74	123
DL-3-Hydroxyhexadecanoate	1.24 ± .37	3.22 ± .40	160

* Mean ± S.E.$_M$ for 5 experiments.
† See Table II for derivation.

strates by liver mitochondria from diabetic rats significantly greater than that seen with normal tissue. A greater carnitine effect in diabetic tissue might have been masked by large increases in fatty acid oxidation to carbon dioxide as a result of diabetes. Suprisingly, however, under our experimental conditions mitochondrial oxidation to carbon dioxide of all fatty acids except 3-ketopalmitate, and the production of acetoacetate from oxidation products, were not decreased by diabetes. It is possible that the cellular disruption and washing procedures required to isolate liver mitochondria removed factors responsible for the elevated hepatic oxidation of fatty acids which can be observed in intact animals and in relatively intact liver preparations(14); we have found no literature report of elevated fatty acid oxidation in hepatic mitochondrial preparations from diabetic animals. Production of the aforementioned hypothetical factors might be induced by the high tissue levels of free fatty acids known to result from increased transfer of fatty acids from adipose tissue to the liver in experimental diabetes.

We have previously noted a very high level of mitochondrial oxidation of 3-ketohexadecanoate relative to that of palmitate and the other two β-oxidation intermediates, and have reported a 2-fold increase in the oxidation of this derivative in diabetes(2). However, in contrast to this initial report(2), subsequent studies in which experimental conditions were more rigorously controlled, failed to reveal increased oxidation of 3-ketohexadecanoate by liver mitochondria from diabetic rats.

We have no explanation for the lack of a carnitine effect on the mitochondrial oxidation of 3-ketohexadecanoate (Table I, III, IV). Considering the fact that the rate of oxidation of 3-ketohexadecanoate by liver and heart mitochondria was about 200 times greater than that of the other 3 substrates, it is possible that this compound rapidly and freely diffuses through mitochondrial membranes, and that no carnitine-mediated carrier mechanism is required to facilitate transport.

In the light of the present results, we may anticipate that specific or general transferases exist for the formation of the carnitine esters of 2-hexadecenoic and 3-hydroxyhexadecanoic acids from the corresponding CoA derivatives. We are presently engaged in the chemical synthesis of these derivatives, and in a search for specific transferases.

Summary. The effects of carnitine and BSA on the oxidation to CO_2 and conversion to acetoacetate of [1-^{14}C] palmitate, [1-^{14}C] *trans*-2-hexadecenoate, [1-^{14}C] DL-3-hydroxyhexadecanoate and [1-^{14}C] 3-ketohexadecanoate were examined in liver mitochondria from normal and diabetic rats and in heart muscle mitochondria from normal animals. Carnitine stimulated the oxidation of all substrates except the 3-keto intermediate by normal liver and heart muscle mitochondria; BSA increased basal oxidation and augmented the carnitine effect on the oxidation of these same compounds in both tissues. Carnitine increased the conversion of palmitate and the unsaturated and hydroxy derivatives to acetoacetate in liver mitochondria; BSA greatly increased both basal and carnitine-stimulated acetoacetate production; no acetoacetate accumulated under any conditions in heart mitochondria. Diabetes did not significantly influence the oxidation of, or the accumulation of acetoacetate from, any of the 4 substrates tested in liver mitochondria, nor was the effect of carnitine greater than normal

in tissue obtained from diabetic rats.

The skillful technical assistance of Mrs. JoAnn Lohsen is gratefully acknowledged.

1. Jones, J. A., Blecher, M., J. Lipid Res., 1966, v7, 422.
2. ———, J. Biol. Chem., 1965, v240, 68.
3. Fritz, I. B., Adv. Lipid Res., 1963, v1, 285.
4. Bremer, J., J. Biol. Chem., 1962, v237, 2228.
5. Bressler, R., Katz, R., ibid., 1965, v240, 622.
6. Fritz, I. B., Acta Physiol. Scand., 1955, v34, 367.
7. Fritz, I. B., Kaplan, E., Yue, K. T. N., Am. J. Physiol., 1962, v202, 117.
8. Norum, K. R., Biochem. Biophys. Acta, 1965, v98, 652.
9. Hollis, V. W., Jr., Blecher, M., Fed. Proc., 1966, v25, 838.
10. Fritz, I. B., Kaplan, E., in H. Peeters, ed. Protides of the Biological Fluids, VII Colloquium, Elsevier, Amsterdam, 1960, 252.
11. Fritz, I. B., Adv. Enzyme Regulation, 1963, v1, 286.
12. Chaikoff, J. L., Goldman, D. S., Brown, G. W., Jr., Dauben, W. G., Gee, M., J. Biol. Chem., 1951, v190, 229.
13. Nugteren, D. H., Biochem. Biophys. Acta, 1965, v106, 280.
14. Langdon, R. G., in K. Bloch, ed., Lipide Metabolism, J. Wiley, N. Y., 1960, 238.

Received May 1, 1967. P.S.E.B.M., 1967, v125.

Effect of Vinblastine and 4-Amino-N^{10} Methyl Pteroyl-Glutamic Acid On the Erythropoietin Responsive Cell.* (32314)

GEORGE HODGSON
Facultad de Ciencias, Universidad de Chile, Santiago

Bruce and McCulloch(1) have postulated two kinds of stem cells: a) one capable of forming spleen colonies and reestablishing hemopoiesis in a lethally irradiated host(1), henceforth referred to as CFU, and b) cells that respond to erythropoietin, referred to here as ERU (erythropoietin responsive units) derived from the colony forming cells mentioned in a). Porteous and Lajtha, however, are of the opinion that there is no good reason to postulate two kinds of cells(2). Bruce *et al* have shown, using chemotherapeutic agents such as Vinblastine and Aminopterin, which kill cells in cycle(3), that few of CFU in marrow are in generation cycle. On the other hand, the effects of low doses of irradiation and colchicine suggest that the ERU respond to EP shortly after mitosis(4). Vinblastine has been shown by Valeriote *et al* to be useful for the study of the cell cycle *in vivo*(5). This drug kills only cells passing through mitosis(6), has a biological half life of 3.5 hours(7) and a discontinuous dose response curve with a critical dose of 5 μg for mice, below which no cells die, above which 100% mortality occurs(7). Vinblastine thus seems a valuable tool to determine whether ERU are in cycle before being exposed to EP and within what time after exposure to EP they go through mitosis. The present paper reports the results of effects of Vinblastine† (Velban) and 4-Amino-N^{10} methyl pteroyl-glutamic acid‡ (Methotrexate) a drug also known to affect cells in cycle(3), on the response to EP of mice with transfusion polycythemia.

Materials and methods. Ten-to-twelve-weeks-old 18-20 g female $B_{10}D_2$ mice, maintained in the Dept. of Biology, School of Medicine, University of Chile, were used as recipients. Each group contained at least 5 mice. They were transfused with ½ ml of washed erythrocytes, intravenously on day 0 and 1. Five units of erythropoietin were injected subcutaneously on day 6, 0.1 μc ^{59}Fe ferric citrate (specific activity >5 μc/μg) was injected on day 8. The mice were bled on day 9 and radioactivity of washed erythrocytes and micro hematocrits measured. Only mice with heart

* This work was partially supported by a Grant from U. S. Atomic Energy Commission to Univ. of Chile, NYO-AT (30-1)-2488-10.

† Generous gift from Eli Lilly and Co., Indianapolis.

‡ Generous gift from Lederle Lab., Pearl River, N. Y.