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THE EFFLUX OF L-CARNITINE FROM CELLS IN CULTURE (CCL 27)

PER MØLSTAD

Institute for Nutrition Research, Blindern, and Institute for Research in Internal Medicine, Rikshospitalet, University of Oslo (Norway)

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Summary

The efflux of L-[^{3}H]carnitine was studied in cells from an established cell line from human heart (Girardi human heart cells, CCL 27). The cells were loaded with 4 μmol/l L-[^{3}H]carnitine for 1 or 24 h, and the efflux of radioactivity into the medium was measured. The amount of intracellular L-[^{3}H]-carnitine retained was expressed as a function of time. The results were fitted to an exponential equation, from which efflux rate constants were computed.

Increasing the extracellular concentration of butyrobetaine, L-carnitine, D-carnitine, betaine, DL-norcarnitine or 3-dimethylamino-2-hydroxypropionic acid each increased the observed efflux. This is most likely due to accelerated exchange diffusion. The substrate specificity of this accelerated exchange diffusion is different from what previously has been found in competitive uptake studies of L-carnitine. L-Carnitine was preferentially released to L-acetylcarnitine, and blocking the sulfhydryl groups with 5,5-dithiobis-(2-nitrobenzoic acid) increased the efflux.

Introduction

The intracellular concentration of L-carnitine is substantially higher than the plasma concentration in most tissues [1,2]. An active transport mechanism for the uptake of L-carnitine has been described in an established cell line from human heart (CCL 27) [3—5], in rat skeletal muscle [6,7] and isolated rat liver cells [8]. The rate of transport and the intracellular concentration of L-carnitine are subject to metabolic and hormonal regulation [5,9—14]. This regulation is defective, however, resulting in low level of intracellular carnitine in

Abbreviation: DTNB, 5,5-dithiobis-(2-nitrobenzoic acid).

guinea pigs injected with diphteria toxin [15], in patients treated with hemodialysis for chronic renal insufficiency [16], and in muscular carnitine deficiency syndrome [17].

The rate of efflux of intracellular carnitine would affect the intracellular concentration and is thus of importance in the regulation. Efflux has previously been described by Rebouche [6] in rat skeletal muscle. The present study was undertaken to investigate the efflux mechanism in the established cell line where the mechanism for uptake of L-carnitine previously is characterized [3–5].

Materials and Methods

The materials used and methods employed in measuring the uptake of L-[*Me*-^{3}H]carnitine in Girardi human heart cells (CCL 27) have previously been described [3–5,9].

The efflux of carnitine was studied by loading the cells with L-[^{3}H]carnitine (4 μmol/l) for 1 or 24 h. The medium was then removed, and the monolayers were washed six times with 3 ml medium devoid of carnitine and serum. 10 ml of fresh medium was added, and the release of L-[^{3}H]carnitine into the medium was followed by removing aliquots of 200 μl medium after 0, $\frac{1}{2}$, 1, 2, 3 and 4 h. The medium was then removed and the cells were washed three times with 3 ml fresh medium. Thereafter the monolayers were exposed to a 0.02% solution of trypsin for 30 s at room temperature, and subsequent to the removal of the trypsin solution, incubated for 10 min at 37°C. The cells were then dissolved in 10% trichloroacetic acid and heated to 100°C for 30 min. The radioactivity measured in the trichloroacetic acid was taken as the amount present intracellularly at the end of the experiment [3].

The efflux of L-[^{3}H]carnitine is described by the amount of L-[^{3}H]carnitine remaining intracellularly with time, which is calculated as percent of the concentration at zero time. The intracellular zero time concentration was calculated from the sum of radioactivity present in the cells at the end of the experiment and the amount that had been released into the medium. The zero time concentration of L-[^{3}H]carnitine in the medium was measured in each incubation (about 0.01 μmol/l or 350 cpm/ml) and subtracted from the amount subsequently measured in the medium.

The results obtained for the decrease of intracellular L-[^{3}H]carnitine with time, were fitted to an exponential equation by the least-squares method (ln % intracellular L-[^{3}H]carnitine = $100 - k \cdot t$; t = time). The efflux rate constant (k) was used as an expression of rate of efflux in comparisons between samples.

The distribution of radioactivity intracellularly and in the medium between free carnitine and acylcarnitines was assessed as described by Christiansen and Bremer [8]. On thin-layer chromatography in this system L-[^{3}H]carnitine had a mean R_F value of 0.10, L-[^{3}H]acetylcarnitine 0.20 and a third unidentified radiolabeled acylcarnitine 0.30.

Results

The release of L-[^{3}H]carnitine from the cells with time is shown in a semilog plot in Fig. 1. When the concentration of L-carnitine in the extracellular

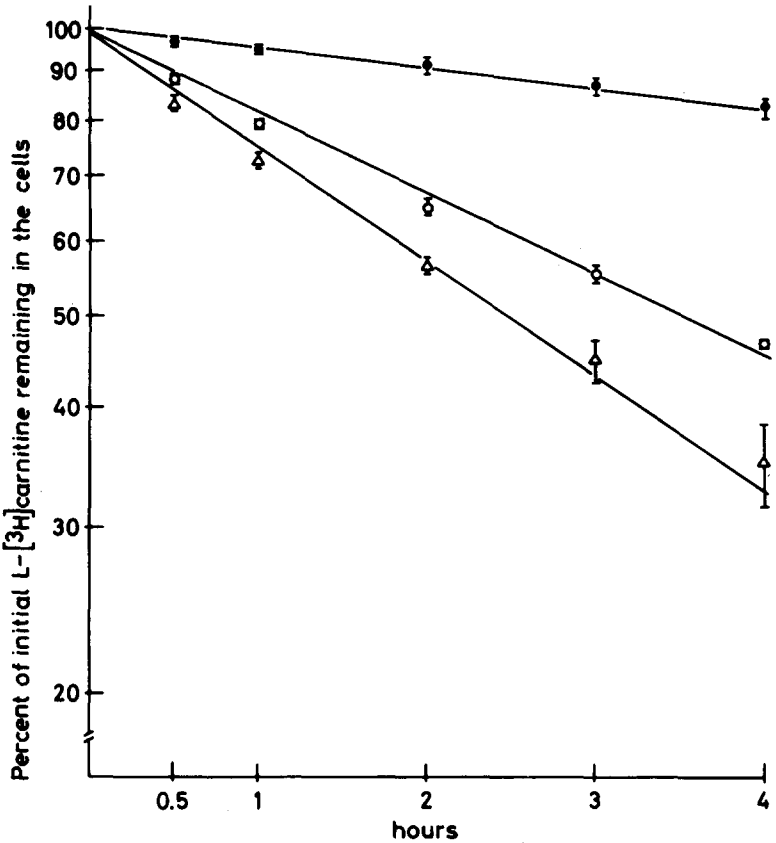

Fig. 1. The effect of increasing the concentration of unlabeled L-carnitine in the extracellular medium on the efflux of L-[^{3}H]carnitine from preloaded cells (4 μmol/l L-[^{3}H]carnitine for 1 h). ●———●, efflux into medium without any addition; ○———○, medium with 10 μmol/l L-carnitine; △———△, medium with 100 μmol/l L-carnitine. The experiment was performed and calculations were done as stated in Materials and Methods. The results are means of three to four separate incubations, and the vertical bars represent S.D. The efflux-rate constants were: medium without any addition, 0.0476; medium with 1 μmol/l L-carnitine (not shown in the figure), 0.0562; medium with 10 μmol/l L-carnitine, 0.1892, and medium with 100 μmol/l L-carnitine, 0.2570. The correlation coefficients of the results to the computed equations were above 0.96 in all cases. The zero time concentration of intracellular L-[^{3}H]carnitine (mean ± S.D.) was 15.6 ± 1.4 pmol · μg^{-1} DNA, and the incubations contained 190—335 μg DNA (1600—2800 μg protein).

medium was increased from zero to 100 μmol/l, the efflux-rate constant increased five times (Fig. 1). In another experiment a further increase in the concentration of L-carnitine merely resulted in marginal changes in the kinetic parameters (not shown).

The efflux rate constant increased about four times when the temperature during the efflux was increased from 4°C to 37°C (Fig. 2). Loading the cells with 4 μmol/l L-[^{3}H]carnitine for 1 or 24 h, did not affect the rate of efflux substantially. The efflux-rate constant merely decreased from 0.113 to 0.081 (not shown). The intracellular concentration of L-[^{3}H]carnitine at zero time was (mean ± S.D.) 9.9 ± 1.1 pmol · μg^{-1} DNA after 1-h load, and 24.7 ± 1.9 pmol · μg^{-1} DNA after a 24-h load.

The molecular specificity of the increase in the efflux of L-[^{3}H]carnitine

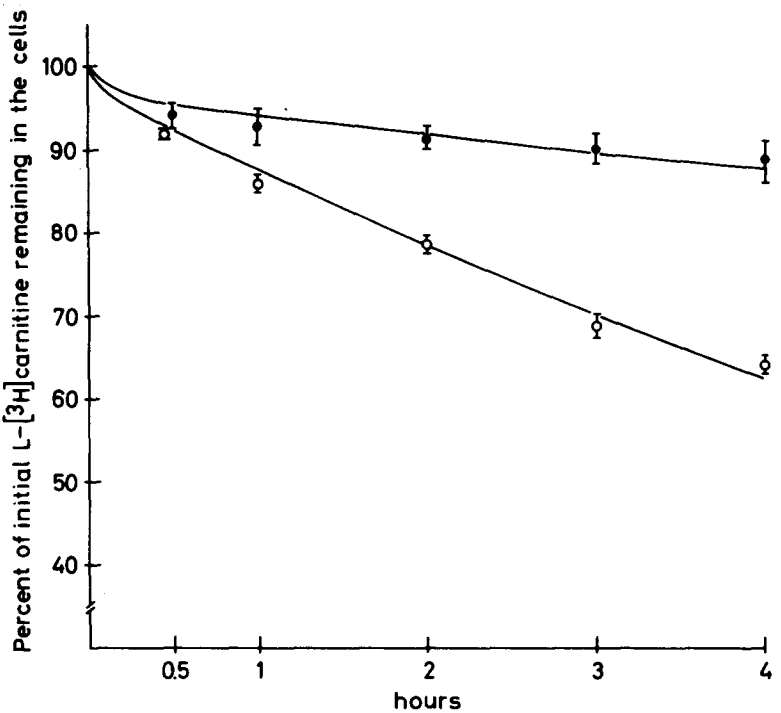

Fig. 2. The efflux of L-[^{3}H]carnitine at 4° C (●——●) and 37° C (○——○) plotted arithmetically. The cells were loaded with 4 μmol/l L-[^{3}H]carnitine for 1 h. The experiment was performed and computed as stated in Materials and Methods. Each result is the mean of three incubations, and vertical bars represent S.D. The efflux-rate constants were: 4° C, 0.0238; 25° C (not shown in figure), 0.0320, and 37° C, 0.1095. The correlation coefficients of the data to the computed equations were: 0.84, 0.97 and 0.99, respectively. The means ± S.D. zero time concentration of intracellular L-[^{3}H]carnitine was 16.3 ± 1.8 pmol · μg^{-1} DNA, and the incubations contained 75—115 μg DNA (600—1000 μg protein).

observed with increasing concentrations of unlabeled L-carnitine in the medium was studied. This was accomplished by adding compounds structurally related to carnitine to the medium (Table I). The efflux-rate constant in presence of butyrobetaine was significantly higher than what was obtained with the equivalent amount of L-carnitine. L-acetylcarnitine, D-carnitine and betaine produced kinetic parameters indistinguishable from those of L-carnitine. In the presence of DL-norcarnitine and 3-dimethylamino-2-hydroxypropionic acid the rate constants were significantly lower than obtained with the equivalent amount of L-carnitine. However, the constants were greater than what was produced by medium without any addition.

It has previously been shown that the uptake of L-carnitine in this system depends upon the presence of free sulfhydryl groups [3], and thus is inhibited by 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman reagent). The efflux of L-[^{3}H]carnitine in presence of 1.5 mmol/l DTNB was increased, both by DTNB alone and in combination with L-carnitine (Fig. 3). The abrupt fall in the intracellular content of L-[^{3}H]carnitine after 3 h in cells exposed to 1.5 mmol/l DTNB, does suggest a toxic effect. No change, however, was found in the percentage of cells excluding trypan blue (>95%).

TABLE I

RATE CONSTANTS FOR THE EFFLUX OF L-[^{3}H]CARNITINE WITH DIFFERENT COMPOUNDS ADDED TO THE EXTRACELLULAR MEDIUM

The efflux of L-[^{3}H]carnitine was measured after loading of the cells with 4 μmol/l L-[^{3}H]carnitine for 1 or 24 h. Different compounds were added to the extracellular medium as indicated in the table, and the efflux-rate constants were computed as stated in Materials and Methods. The data represents means from three to four separate incubations. The correlation coefficients of the results to the computed equations were above 0.97 in all cases. The efflux rates produced by the different compounds were compared with that obtained by equimolar amounts of L-carnitine by means of Wilcoxon's signed rank test. The mean ± S.D. zero time concentration of intracellular L-[^{3}H]carnitine was 7.0 ± 2.2 pmol · μg^{-1} DNA, and the incubations contained 50—315 μg DNA (425—2600 μg protein). The results obtained with medium without any addition (designated: control) was included as a base-line reference in each experiment.

Experiment No.	Added to the extracellular medium	Efflux-rate constants (k)
1	0, control	0.1067
	10 μmol/l L-carnitine	0.1585
	10 μmol/l betaine	0.1668
2	0, control	0.0480
	100 μmol/l L-carnitine	0.1063
	100 μmol/l butyrobetaine	0.1549 *
3	0, control	0.0576
	100 μmol/l L-carnitine	0.1687
	100 μmol/l L-acetylcarnitine	0.1827
	100 μmol/l D-carnitine	0.1677
4	0, control	0.0374
	100 μmol/l L-carnintine	0.0831
	100 μmol/l DL-norcarnitine	0.0645 *
	100 μmol/l 3-dimethylamino-2-hydroxypropionic acid	0.0579 *

* Significant difference ($P < 0.001$).

TABLE II

THE RELATIVE DISTRIBUTION OF RADIOACTIVITY BETWEEN CARNITINE AND DERIVATIVES

The table shows in percent the relative distribution of radioactivity in the cells and in the medium into which the efflux of L-[^{3}H]carnitine has taken place for 4 h. The cells were loaded with 4 μmol/l L-[^{3}H]-carnitine for 1 or 24 h. The total amount of radioactivity in 19 separate incubations was (mean ± S.D.) 8.9 ± 4.8 pmol · μg^{-1} DNA in the cells, and 0.3 ± 0.02 μmol/l in the medium. The thin-layer chromatography was performed as described by Christiansen and Bremer [8]. The results are means ± S.D. from four to five separate incubations. The intracellular distribution of radioactivity showed the same pattern whether measured at 0, 4 or 6 h after the termination of preload. This probably indicates that intracellular equilibration between carnitine and derivatives is rapid process in comparison to the efflux. Neither was the intracellular distribution affected by the duration of preload (1 or 24 h). The difference in the relative amount of L-acetylcarnitine between the cells and the medium is significant ($P = 0.02$, Wilcoxon's rank sum test).

	Carnitine (%)	Acetyl-carnitine (%)	Acyl-carnitine (%)
Cells	47 ± 16	48 ± 12	5 ± 4
Medium	76 ± 14	22 ± 14	3 ± 2

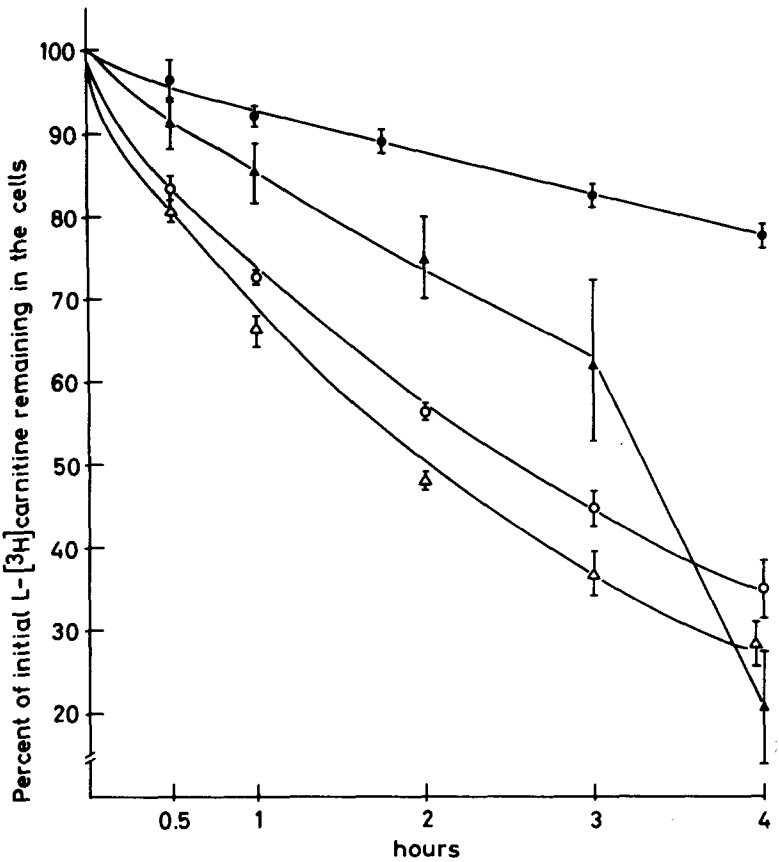

Fig. 3. The effect of DTNB on the efflux of L-[^{3}H]carnitine from preloaded cells (4 μmol/l L-[^{3}H]carnitine for 1 h), plotted arithmetically. ●———●, efflux into medium without any addition; ○———○, medium with 100 μmol/l L-carnitine; ▲———▲, medium with 1.5 mmol/l DTNB, and △———△, medium with 0.5 mmol/l DTNB and 100 μmol/l L-carnitine. The results are means from three to four separate incubations from two different experiments, and vertical bars represent S.D. The experiments were performed and calculations were done as stated in Materials and Methods. The efflux rate constants were: medium without any addition, 0.0456; medium with 1.5 mmol/l DTNB, 0.1536; medium with 100 μmol/l L-carnitine, 0.2571; medium with 0.5 mmol/l DTNB and 100 μmol/l L-carnitine, 0.3130. The results obtained after 4 h with 1.5 mmol/l DTNB in the medium were omitted from the calculation of the rate constant. The correlation coefficients of the data to the computed equations were above 0.83 in all cases. DTNB increased the rate of efflux significantly both when present alone and in combination with 100 μmol/l L-carnitine in the medium ($P < 0.0005$, Wilcoxon's signed rank test). The zero time intracellular concentration of L-[^{3}H]carnitine (mean ± S.D.) was 12.6 ± 5.2 pmol · μg^{-1} DNA. The incubations contained 230—325 μg DNA (2000—2800 μg protein).

The cells preferentially released L-carnitine to L-acetylcarnitine to the medium (Table II), as evidenced by the significant decrease in the relative amount of L-acetylcarnitine in the medium.

Discussion

When efflux took place into medium without any addition of unlabeled L-carnitine, the concentration of L-[^{3}H]carnitine increased from about 0.01 μmol/l to 0.03 μmol/l (not shown). From previously reported results [3] the

amount of L-[^{3}H]carnitine reentering the cells can be estimated. This amounts to 5—25% of the efflux. Thus it seems that under the reported conditions one observes mainly the efflux. By adding unlabeled L-carnitine to the medium in excess, reentry of the radioactive isotope would be abolished (Fig. 1, Table I).

It should be noted that the efflux-rate constants increase with increasing amount of unlabeled L-carnitine in the medium. This could be due to inhibition of reentry or accelerated exchange diffusion. If blocking of reentry should explain the effect, one would not expect to find a difference in the kinetic parameters between 10 μmol/l and 100 μmol/l L-carnitine in the medium. The ratio between unlabeled and radiolabeled L-carnitine would be 300 and 3000, respectively. Both these ratios would be more than sufficient to completely inhibit reentry of the radiolabeled isotope. Thus, some of the increase must be due to accelerated exchange diffusion.

The structural specificity in inducing accelerated exchange diffusion (Table I), is different from what was found during competetive uptake studies [3,4]. During uptake studies butyrobetaine and L-carnitine seem to have identical affinity for the carrier mechanism. Concerning efflux however, butyrobetaine gives rise to a greater efflux-rate constant than L-carnitine (Table I). D-carnitine and betaine both have a lower affinity for the uptake mechanism than L-carnitine (50% inhibiting concentrations on the uptake of 2 μmol/l L-[^{3}H]carnitine were 20 μmol/l and 90 μmol/l, respectively) [4]. In accelarated exchange diffusion, however, they produced rate constants indistinguishable from that of L-carnitine (Table I). Compounds with a dimethylamino group do not compete with L-carnitine for the entry [4]. They do, however, produce accelerated exchange diffusion (Table I). This fact seems to rule out trapped reentry as the sole explanation for the increased efflux induced by L-carnitine and related compounds. The observed accelerated exchange diffusion indicates that the efflux of L-carnitine is carrier-mediated. The differences in structural specificity reported above could imply that the uptake and release transport mechanisms are not identical.

During efflux studies, control of the intracellular concentration of the compound studied is often difficult. In the reported experiments two different loading conditions were used, resulting in a 2.5-fold variation in the intracellular concentration of L-[^{3}H]carnitine. The effect on the efflux was, however, small; about 30% reduction in the efflux-rate constant (from 0.1131 to 0.0811). This could represent a saturation in the rate of efflux imposed by the highest load. Alternatively, it might merely indicate that 1 h is too short to obtain complete equilibrium between intracellular pools. The results of Ramsay and Tubbs [18] would support the latter explanation. A rough estimate from their results indicates that the amount of L-[^{3}H]carnitine taken up during a 1-h load would need about 40 min for exchange across the mitochondrial membrane.

Free sulfhydryl groups are essential for the uptake of L-[^{3}H]carnitine [4]. Blocking the SH groups with DTNB, caused a moderate increase in the efflux rate constants (Fig. 3). This increase in efflux is not the basis for the observed inhibition in entry since under the conditions employed during the uptake studies the efflux of L-[^{3}H]carnitine is insignificant in comparison to the entry [3]. The cell membrane is impermeable to DTNB [19], its effect must there-

fore be exerted on the membrane. The mechanism for this effect is not clear. It is, however, reported that an increase in the efflux of Na^+ and K^+ occurs by SH-blocking agents in erythrocytes [20]. This could indicate that SH groups are involved in the regulation of the permeability of the cell membrane.

The main difference between the intra- and extra-cellular distribution of L-carnitine and its derivatives, is a reduction in the relative amount of L-acetylcarnitine released into the medium (Table II). This is in contrast to what is found in the studies of entry, where the kinetic parameters for L-carnitine and L-acetylcarnitine were nearly identical [3,4].

The tissue concentration of carnitine is altered in a number of conditions [21]. Studies of both entry and release of L-carnitine will be needed in order to understand how these changes arise.

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