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Enhanced uptake of carnitine by perfused rat liver following starvation

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Previously, the release of carnitine from the perfused rat liver was found to be protein-mediated, dependent on the nutritional state but not on metabolic energy. Further, it was shown to exceed the physiological demand by about 10-fold (Sandor et al. (1985) Biochim. Biophys. Acta 835, 83–91). In the present study the uptake of carnitine by perfused rat liver has been investigated. (1) The liver tissue and the perfusate were in equilibrium when the carnitine concentration in the perfusate was close to 45 μM, physiological in the rat plasma. Under this condition, when no net carnitine transport occurred, an unidirectional uptake of L-[^{3}H]carnitine was observed. Quantitatively, the uptake rate was 355 ± 60 (S.D.) nmol/h per 100 g body weight at 45–50 μM perfusate concentration. This uptake capacity balances the previously reported excessive release (Sandor et al., op. cit.). On this basis we propose that a futile release/uptake cycle operates in carnitine transport across the liver cell membrane. (2) Liverse of 24-h starved rats took up L-[^{3}H]carnitine at 56% higher rate from the perfusate (75 μM) than livers of fed rats. Kinetic analysis revealed that fasting caused a decrease in K_m value from 4.22 mM to 2.59 mM, whereas V_{max} remained practically unchanged, average 0.95 μmol/min per 100 g body weight. D-[^{3}H]Carnitine was transported at the same rate as L-carnitine and underwent the effect of fasting as well. (3) The uptake was partially inhibited by 1 mM 2,4-dinitrophenol and 5 mM KCN, showing its dependency on metabolic energy. If Li^+ replaced Na^+ a strong inhibitory effect (to 20% of control) was observed, which suggests a co-transport of carnitine with Na^+. Mersalyl, an SH reagent, had no effect on the uptake, whereas it practically abolished the release of carnitine from the perfused livers. This observation suggests that the inward and outward transport of carnitine are mediated by two different proteins.

Introduction

It has been well documented the rat liver in the fasting state accumulates carnitine (i.e., L-(−)-carnitine: 3-hydroxy-4-*N*-trimethylammoniobutanoate) [1–3], an essential substance for fatty acid oxidation and ketogenesis. The increase of carnitine content on a g liver basis is undoubted, but the mechanism(s) whereby rat liver concentrates carnitine during fasting is not completely understood. Whereas some workers found an absolute rise in liver carnitine content [2,3], others found that the increased concentration could be accounted for by loss of liver mass [1]. We pointed out earlier [3] that shrinkage of the liver during starvation cannot be the cause by itself. An elevated concentration in liver should equilibrate with the serum at the earlier fed level, unless the kinetic parameters of cellular transport are changed. In a previous work [3] we demonstrated

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a decreased rate of carnitine release from the liver during starvation, which is due to a decrease of rate constant (to 5.0 min^{-1} from 8.4 min^{-1}). In the present paper, the uptake side of carnitine transport has been investigated. As will be demonstrated, the enhanced uptake also contributes to developing the increased liver carnitine concentration seen in starvation.

The uptake process, characterized hereafter, deserves attention in two points when compared to the efflux. In rat, the liver synthesizes carnitine and supplies the whole organism with that. However, the efflux of carnitine from the liver [3] exceeds several times the estimated physiological demand and rate of biosynthesis [1,4,5]. Thus, most of the released carnitine has to reenter the liver. A question to be answered is whether or not the influx rate matches the excessive release? In a qualitative sense, the uptake process will be characterized through its sensitivity to inhibitors. Comparing these properties of influx to those of the efflux process we can obtain further information about the transport mechanism: do the uptake and release share a common protein?

Materials and Methods

Male Wistar rats (200–250 g) were used in all experiments and fed commercial laboratory chow ad libitum. Fasted rats were deprived of food at 8:00 a.m. the day before the experiment.

Materials

L-Carnitine, internal salt, was a kind gift from Sigma-Tau, Rome. D,L-[*methyl*-^{3}H]Carnitine and [1-^{14}C]acetyl-CoA were purchased from Amersham. Inulin [^{14}C]carboxylic acid from Amersham was rechromatographed on a Sephadex G-25 column before use. Acetyl-CoA was prepared as described [6]. L-[^{3}H]Carnitine was prepared from the commercially available racemic mixtures by paper chromatography after the enzymatic acylation of the L-isomer with pripionyl-CoA [3,7]. The separation of propionyl-L-carnitine from D-carnitine and the subsequent alkaline hydrolysis resulted in D-[^{3}H]carnitine and L-[^{3}H]carnitine. The overall yield was 90% with 99% purity as determined by TLC. Carnitine acetyltransferase and 3-hydroxybutyrate dehydrogenase were obtained from Boehringer-Mannheim. All other chemicals were market-available analytical grade compounds.

Experimental procedures

Livers were perfused with 30 ml perfusion fluid as described by Mortimore [8] with some modifications [3]. Samples of perfusion fluid were taken at time intervals shown in the figures, and of livers at the end of perfusion. Perfusate and liver samples were assayed for total carnitine after alkaline hydrolysis by modified enzymatic analysis [3] originally described by Cederblad [9]. To determine the unidirectional uptake rate we introduced L-[^{3}H]carnitine (usually 1000 cpm/nmol) into the perfusate and measured radioactivity in the perfusion fluid. To express the changes in nmol the initial specific activity was used. For calculations of uptake rates we used the initial straight part of the curves between 5 and 15 min. (The diluting effect of released carnitine on the specific activity was neglected because it could affect the calculation less than 10%.) Ketone bodies were determined in the deproteinized extracts by described methods [10,11] (the term ketone bodies refers to the sum of acetoacetate and 3-hydroxybutyrate).

Extracellular space of perfused livers was determined with [*carboxy*-^{14}C]inulin in separate experiments, tissue total water was determined by drying as described [3] and the intracellular water was calculated by subtraction. Application and standardization of Mersalyl was also described earlier [3]. Radioactive determinations in 10 ml toluene/Triton X-100 (2 : 1, v/v) scintillant were performed using Beckman LS 230 counter. ^{3}H activities were measured in separate aliquots in absence of ^{14}C. ^{14}C activities were measured by using narrow window if tritium activity was present.

Terminology

'Uptake' or 'unidirectional uptake' means the influx. 'Release' means the efflux. 'Net uptake' means the resultant inward transport of the concomitant influx and efflux. For example, when the influx and efflux are equal not net uptake occurs.

Results

Fed and fasted state

The carnitine concentration in the perfusate

and tissue were compared during perfusion of livers from fed and 24-h fasted rats. In respect to ketone body production the perfused liver preserved the in vivo fed-fasted relationship. The 'fed' livers produced 18 μmol, 'fasted' livers produced 80 μmol total ketone bodies per h per 100 g body weight (data not shown), consistent with the value reported in the literature [12].

Carnitine concentrations in the perfusate during the perfusion are shown in Fig. 1. When the initial carnitine concentration in the perfusate was 45 μM (considered physiological), this level remained constant during the 2 h of perfusion (Fig. 1, set I). This steady-state concentration is the result of the concommitant uptake and release. The steady state was achieved at a lower perfusate concentration by livers from fasted animals (Fig. 1). Hepatic carnitine content at the end of perfusion was 170 nmol/g and 356 nmol/g in fed and starved rats, respectively. These levels correspond to 307 μM and 622 μM intracellular concentrations, when calculated on intracellular water basis. (The intracellular water content increased slightly from 552 to 572 μl/g wet weight during starvation, in accordance with other reports [8]). The liver-to-perfusate concentration ratios of carnitine at equilibrium were 6.9 vs. 18.2 when livers derived from fed and fasted animals, respectively. In vivo the liver/serum ratio undergoes the same shift (because the liver develops its higher concentration at practically unchanged serum level). Thus, the recirculating perfusion system maintains the liver-serum relationship with respect to the carnitine concentration, as well. When perfusate carnitine concentration was higher, 78–80 μM, livers took up carnitine, approaching the steady-state condition (Fig. 1, set II). The rate of net uptake in fasted state was increased by 62.5% when compared with the rate seen in livers from fed rats. This apparent increase in the net uptake raised two questions. First, because the decreased release in the fasted state [3] contributes to this effect, what was the effect of starvation on the unidirectional uptake process? Second, might this effect be due to metabolism, i.e., enhanced esterification of carnitine?

To answer the first question, we introduced L-[^{3}H]carnitine into the perfusate to determine the unidirectional uptake. Fig. 2 shows the unidirectional and net uptake simultaneously in both nutritional states. The divergence between the net and unidirectional curve is due to the concomitant release. The unidirectional uptake rates were 36 and 56 nmol/min per liver by livers from fed (Fig.

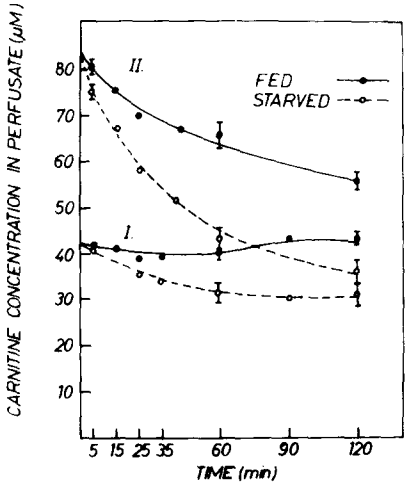

Fig. 1. Total carnitine concentration in perfusate during recirculating perfusion of fed and fasted rats. Livers were 12.5 ± 0.6 g and 9.4 ± 0.5 g at the end of perfusion in the fed and fasted state, respectively. (In this and all other experiments the pre-fasting body weights were the same in the two groups.) Values presented in the figure are means of four experiments. For clarity, S.E. is shown by bars only at 60 and 120 min.

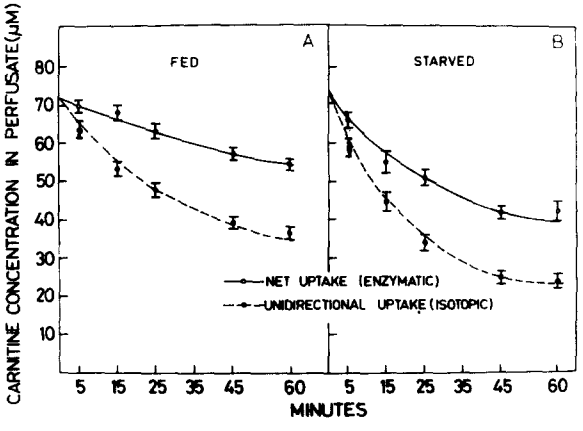

Fig. 2. (A, B) Unidirectional and net uptake of carnitine in fed and fasted states. Rat livers were perfused with 30 ml medium as described in Materials and Methods. Livers were 10.5 and 7.8 g in the fed and starved states, respectively. Unidirectional uptake was measured by the isotopic, net uptake by the enzymatic method, as detailed in Materials and Methods. Values are means ± S.E. for four experiments.

2A) and fasted (Fig. 2B) rats, respectively. Thus, 24-h fasting resulted in a 56% increase in the influx of total carnitine. (The comparison on whole liver basis seems to be very resasonable, because the prefasting liver weights were the same in the two groups. Comparison on 100 g body weight basis would have exaggerated the difference by about 10%.)

To answer the second question the uptake of D-[^{3}H]carnitine was also examined. As seen (Fig. 3A) there was no significant difference between the unidirectional uptake of L- and D-carnitine. This finding is consistent with observation made on isolated rat hepatocytes [13]. Further, D-carnitine uptake was also enhanced by starvation (Fig. 3B); the tissue/perfusate ratio increased from 2.09 to 6.20. This observation rules out the possibility that increased esterification of carnitine was responsible for the enhanced uptake seen in the fasted state.

The perfusion model also enabled us to characterize the kinetics of hepatocellular carnitine transport. For this purpose a separate experimental series was performed at the perfusate concentrations shown in Fig. 4. The initial rate of unidirectional uptake was saturable (not shown). Double-reciprocal plots of these data (Fig. 4) showed a decrease in the K_m value from 4.22 mM to 2.59 mM, whereas the V_{max} remained unchanged (average 0.95 μmol · min^{-1} per 100 g prefasting body weight).

Effect of different inhibitors

All experiments with inhibitors were performed using livers of fed animals. The sensitivity of the transport to sulfhydryl reagent was examined by using mersalyl. As shown in Fig. 6C, mersalyl had no effect on unidirectional carnitine uptake. We demonstrated previously that mersalyl strongly inhibits the release of carnitine from the perfused rat liver [3]. Simultaneous measurement of the mersalyl effect on the unidirectional and net uptake enabled us to compare the effect on the uptake and release in the same liver under the same conditions. In Fig. 5A, the unidirectional and net uptake in 'fed' livers are shown. (The divergence between the two curves is due to the

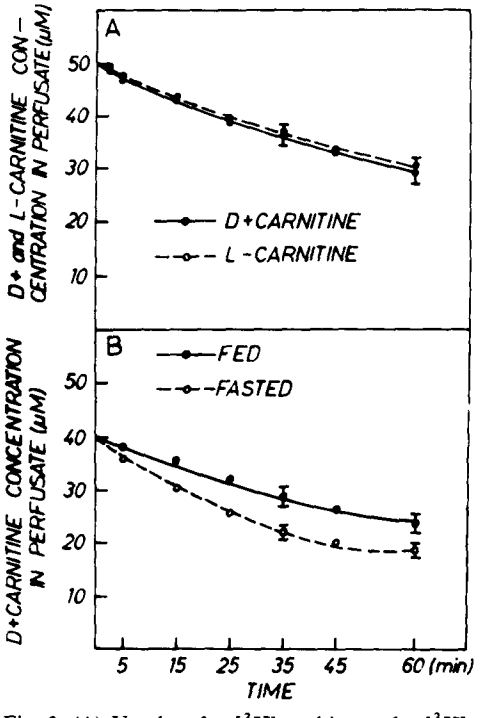

Fig. 3. (A) Uptake of D-[^{3}H]carnitine and L-[^{3}H]carnitine by perfused livers from fed rats. 8.5 ± 0.5 g livers were perfused at concentrations showed on the scale. Values are means of four experiments; S.E. is shown by bars at two points. (B) Uptake of D-[^{3}H]carnitine by perfused livers of fed and starved rats. 9.5 ± 0.5 g fed and 6.5 ± 0.6 g starved livers were perfused as described above. Concentrations of D-carnitine in the liver and the end of perfusion were 68.2 and 133.2 nmol/g in the fed and fasted cases, respectively.

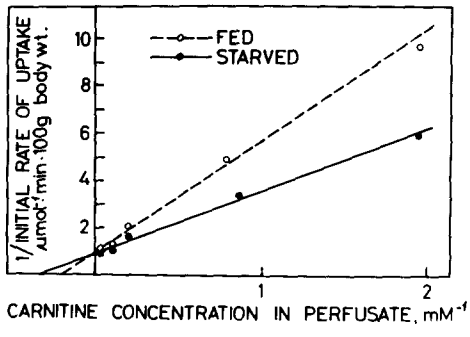

Fig. 4. Double-reciprocal plot of carnitine uptake rate by perfused livers from fed and fasted rats against perfusate carnitine concentration. The unidirectional uptake was measured isotopically; initial rates were assumed as reasoned under Materials and Methods. All points are means for four perfusions. Lines were fitted with least-squares regression. The K_m values: 4.22 mM for fed rats, 2.59 mM for fasted ones. The V_{max} values are 0.92 and 0.98 μmol/min per 100 g body weight.

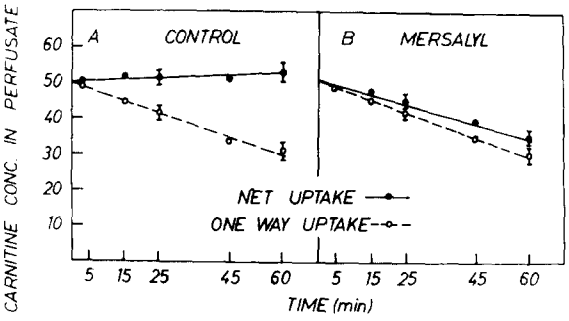

Fig. 5. (A, B) Unidirectional and net uptake of carnitine in the presence (B) and absence (A) of mersalyl. 8.4±0.3 g livers from fed rats were perfused. The effective mersalyl concentration was 380 μM. Values are means for four perfusions, S.E. is shown by bars at important points.

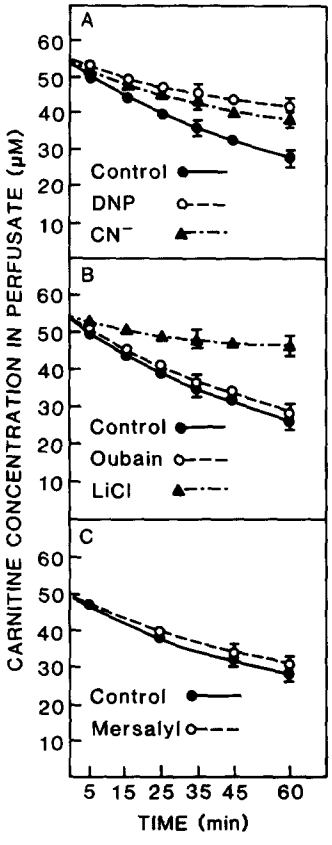

Fig. 6. (A, B, C) Effects of different inhibitors on unidirectional uptake of carnitine. 8.5±0.5 g livers were perfused as described in Materials and Methods. Perfusion medium contained 5 mM KCN, 1 mM 2.4-dinitrophenol, 1 mM ouabain and 380 μM effective mersalyl in different sets of experiments, respectively. In the experiment where the effect of LiCl was examined, the NaCl content of the medium was replaced totally with LiCl. Values are means of four experiments, S.E. is shown by bars at two points.

release of carnitine.) Mersalyl abolished the divergence, that is, abolished the release, while the unidirectional uptake rate was uneffected (Fig. 5B).

Previous studies of carnitine transport by different tissues [13–21] revealed that the uptake process was energy-dependent. Our results, obtained in recirculating perfusion system yielded the same results. Measuring the initial rate of unidirectional uptake of L-[^{3}H]carnitine, significant inhibition by 1 mM 2,4-dinitrophenol and 5 mM KCN was found (Fig. 6A). The 50% and 30% inhibitory effects of 2,4-dinitrophenol and KCN are consistent with results obtained by others on extrahepatic tissues [14–16,18]. Replacement of Na^+ with Li^+ inhibited the inward transport by 80% (Fig. 6B), suggesting a close connection between the Na^+ transmembrane gradient and carnitine transport. Oubain, an inhibitor of (Na^+-K^+)-ATPase, did not affect the uptake (Fig. 6B) and neither did changes in perfusate pH from 6.5 to 8.0 (not shown).

Discussion

At carnitine concentration in the perfusate close to the physiological plasma concentrations (45–50 μM), the perfused liver maintained the perfusate concentration constant. Under this condition, when no net carnitine transport occurred, we observed unidirectional uptake using isotopic techniques (Figs. 1, 2, 5A, 6 control curves). This finding proves that concomitant uptake and release processes are operating in the hepatocellular carnitine transport and that they are balanced under steady-state conditions. In rat, the liver is devoted to supplying the organism with carnitine, and the established daily release from the liver, 9.93 μmol per 100 g body weight [3], exceeds by 4–10-fold the estimated demand. In this work, the uptake of carnitine by rat liver, that is, the re-entry of the released carnitine, has been characterized. Quantitatively, the unidirectional uptake rate average 354 ± 60 (S.D.) nmol/h per 100 g body weight at 45–50 μM perfusate concentration. (Compiled data from Figs. 3, 5, 6. Specific liver weights were 4.79 g/100 g body weight.) (The uptake rate calculated from the kinetic parameters would be somewhat higher (582 nmol/h per 100 g

body wt.). This is fairly consistent, if we consider that the kinetic measurements were performed at another (higher) range of substrate concentrations.) On a daily basis, 8.5 μmol of carnitine were taken up per 100 g body weight, which matches fairly well the previously established release rate. Since this uptake is energy-dependent, this release and uptake cycle can be considered an energy-consuming futile cycle. The significance of this cycle is shown by the fact that the amount of carnitine turning over each day is 10-times higher than the carnitine content of liver. Numerous futile cycles have already been described [22], stressing the ability of these cycles to amplify metabolic signals, if the two opposite processes are simultaneously controlled. We looked at how the hepatocellular carnitine transport is controlled in the fasting state.

Starvation results in a marked increase in liver carnitine on a g wet tissue basis [1–3]. Calculated on an intracellular water basis, the concentrations found in this work were 307 μM and 622 μM in the perfused liver of fed and fasted rats, respectively. Due to the reduced weight of livers from fasted animals, the increase in total liver carnitine (nmol/g per g organ) is smaller (Refs. 2, 3 and this work) or there is no increase [1] compared to the fed state. We stress that the liver shrinkage cannot cause and maintain an increased concentration, that is, the increased liver/plasma ratio. Searching for changes in transport properties, we found that the uptake of carnitine is increased by 56% on the effect of fasting (when starting from 75–80 μM perfusate concentration, Fig. 2). It has also been shown that this increase in transport rate is due to a decrease in K_m value while the V_{max} is slightly changed (Fig. 4). The observation that D-(+)carnitine transport also undergoes the effect of fasting proves that the change is not due to further metabolic conversion (esterification). Earlier [3], we demonstrated a decreased release of carnitine from the perfused livers of fasted animals. Returning to the futile cycle outlined above, the liver takes advantage of release/uptake futile cycle: both directions are controlled in the fasting state in favor of developing and maintaining the higher liver:plasma carnitine ratio (Fig. 1). Presumably, the endocrine background, more exactly, the decreased insulin:glucagon ratio during fasting [2], is responsible for changing the hepatocellular carnitine transport.

Being concentrative, the uptake of carnitine into liver cells is sensitive to metabolic inhibitors is consistence with Bremer's results [13] and other data obtained in extrahepatic tissues [14,16]. The relatively low sensitivity to metabolic inhibitors anticipates that the process is not related directly to ATP utilization but to some other energy-dependent process. In fact, our finding (Fig. 6B) suggests a dependency on an Na^+ gradient. A co-transport mechanism with Na^+ for carnitine is also described in perfused rat heart [16]. (Neither those workers nor ourselves have observed any effect of oubain, probably because carnitine uses the already built up Na^+ gradient for transport.) Mersalyl (SH reagent) did not inhibit the uptake of carnitine (Fig. 6C), but markedly inhibited the release of carnitine (Fig. 5), as, confirming our previous finding [3]. These observations strongly suggest that the inward and outward transport of carnitine through the liver cell membrane are mediated by two different proteins. Alternatively, one carrier protein with two oriented sides may be operating: one is mersalyl-sensitive and the other is not.

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