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Uptake of free choline by isolated perfused rat liver

(lecithin/betaine/phosphorylcholine)

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ABSTRACT **The uptake of free choline by isolated perfused rat liver was characterized. A saturable uptake mechanism [K_a = 0.17 ± 0.07 mM (SD); V_{max} = 0.84 ± 0.16 μmol/min × g dry weight] and a nonsaturable mechanism (through which uptake is proportional to choline concentration in the perfusate) were identified. Most of the choline transported into hepatocytes was converted to betaine, phosphorylcholine, or lecithin. Free choline also accumulated within the intracellular space, suggesting that choline oxidase activity does not always limit choline's uptake by the liver.**

Choline, in the form of the phospholipid lecithin, is an integral component of all membranes and an important constituent of the diet. The intake of choline in the United States usually varies between 100 and 600 mg per day (1). Moreover, pharmacologic doses (grams) of choline and lecithin are administered to humans to treat some neurological diseases presumably arising from deficient central cholinergic tone (2). The basis for this therapy is the finding that plasma choline concentration directly influences acetylcholine synthesis and release in rat brain (3, 4). The increases in plasma choline concentrations seen after oral (3–5) or intravenous (6–8) administration of a large choline dose are small and of short duration. This suggests that high-capacity mechanisms must exist for clearing choline from the blood. The characterization of such mechanisms should be useful in deciding how best to administer choline.

The liver influences plasma choline concentration both by synthesizing choline radicals (in lecithin) via sequential methylation of phosphatidylethanolamine (9, 10) and by removing dietary choline from the portal circulation (11, 12). Choline uptake into the liver has been demonstrated both *in vivo* (6, 8, 13) and in the isolated perfused organ (14–16). In published perfusion studies, initial choline concentrations (1.25 and 2.50 mM) were much higher than physiological levels (0.01–0.02 mM) and no attempt was made to determine the kinetics of the uptake phenomenon. In the present study we have examined the kinetics of free choline uptake in isolated liver by using a wide range of perfusate concentrations (0.01–5 mM). We present evidence that free choline is rapidly taken up into the liver through both a saturable and a nonsaturable mechanism.

METHODS

Animals. Sprague–Dawley rats (Charles River Breeding Laboratories), 150–250 g, were housed in a controlled environment (24°C; 12 hr of light from 0600 to 1800 daily). They were fed Purina chow from 0900 to 1200 hr for 2–3 weeks before being used as liver donors. Water was supplied ad lib.

Closed-Circuit Perfusions. Livers were isolated and perfused by a method reported previously (17). This technique avoids the use of anesthesia and anticoagulant. The perfusate was Krebs–Ringer bicarbonate buffer containing 4% (wt/vol) dialyzed bovine serum albumin (fraction V, fatty acid poor; Miles) and 15 mM glucose equilibrated with 95% O_2/5% CO_2. Oxygen concentration in the effluent perfusate was maintained around 0.2 mM (150 mm Hg; 1 mm Hg = 1.333 × 10^2 Pa) by adjusting the perfusion rate; pH was kept at 7.40 by an automatic titrator. The metabolic integrity of the perfused organ was assessed by determination of the ratios [lactate]/[pyruvate] and [3-hydroxybutyrate]/[acetoacetate] in aliquots of perfusate sampled at 15-min intervals. These ratios reflect the oxidoreduction status of the cytosolic and mitochondrial NADH/NAD^+ couples (18) and are typically 6–10 for [lactate]/[pyruvate] and 0.6–0.8 for [3-hydroxybutyrate]/[acetoacetate] (19). During each 2-hr perfusion experiment, livers were exposed to one, two, or three different initial [*methyl*-^{14}C]choline (40,000 dpm/μmol) concentrations. When more than one concentration was used, the second and third were, respectively, 10 and 100 times the first. The initial amount of choline was added to the perfusate at 30 min, with subsequent amounts added at 30-min intervals. The peak choline concentrations achieved in this series of experiments ranged from 0.02 to 5 mM. During the 30 min after each choline administration, samples of perfusate were withdrawn at 3- *or* 5-min intervals. After 120 min, the liver was removed and quick-frozen (20); the perfusate was recirculated in the apparatus for an additional 15 min in order to check for choline disappearance not mediated by the liver.

Radioactivity of perfusate samples was measured in a Beckman LS7000 liquid scintillation counter. Choline was assayed in an aliquot of the same samples by a radioenzymatic assay with choline kinase and [γ-^{32}P]ATP (21).

Metabolite Determinations. In six of the perfusions, aliquots of perfusate and quick-frozen liver specimens were digested in 2 ml of NCS tissue solubilizer (Amersham) at 50°C overnight, and radioactivity was determined in 15 ml of BetaFluor (National Diagnostics, South Somerville, NJ). Other aliquots were extracted in chloroform/methanol/water, 1:1:1 (vol/vol/vol). The aqueous phase was subjected to paper electrophoresis (22), which separated betaine, choline, and phosphorylcholine. The organic phase was chromatographed on a silica gel plate (Whatman LK5D) with chloroform/methanol/water, 65:30:4 (vol/vol/vol), which separated lecithin, lysolecithin, and sphingomyelin. In both electrophoresis and chromatography, internal standards stained with iodine vapor were used to identify radioactive compounds. Bands were eluted into scintillation vials and radioactivity was determined in 15 ml of Scinti-Verse (Fisher).

Open-Circuit Perfusions. Five livers were perfused at 50 ml/min without recirculation of an albumin-free perfusate. At 15 min, a 10-ml bolus containing [*methyl*-^{3}H]choline (1 mM; 58,000 dpm/nmol) and [*carboxyl*-^{14}C]inulin (0.5 mCi/184 mg; 1 Ci = 3.7 × 10^{10} becquerels) was infused via a syringe pump into the portal vein perfusion line over 4 min. During this infusion, biopsies of liver specimens were taken at 1, 2, 3, and

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4 min and quick-frozen (20). The concentration of intracellular labeled choline was calculated by deducting the amount of extracellular choline present in the inulin distribution volume from the total amount of labeled choline found in liver samples.

***In Situ* Perfusions.** *In situ* short-term liver perfusions without recirculation were performed in fed rats anesthesized with 40 mg of Nembutal per kg (Abbott) and heparinized with 100 units of heparin (Eli Lilly) intravenously. The portal vein was cannulated and the liver was flushed with 20 ml of oxygenated Krebs–Ringer bicarbonate buffer over 1 min. This was followed immediately by 10 ml of the same buffer containing 1 mM [*methyl*-^{14}C]choline (2.8×10^5 dpm/μmol) and 15 μCi of [*G*-^{3}H]inulin (0.5 mCi/mg). Immediately thereafter a lobe of liver was excised and quick-frozen between aluminum blocks that had been cooled in liquid N_2 (20). An aliquot of the frozen liver was digested in NCS tissue solubilizer at 50°C overnight, and radioactivity was measured in 15 ml of BetaFluor. A second portion of the liver was extracted in chloroform/methanol/water and subjected to electrophoresis and chromatography as described above.

RESULTS

Perfusate choline concentrations, measured radioenzymatically or calculated from the radioactivity of the perfusate (dpm/ml divided by the specific activity of the injected [*methyl*-^{14}C]-choline), were identical (linear regression slope = 1.00, $P < 0.01$). Electrophoresis and chromatography of selected perfusate samples showed that 90–95% of the radioactivity could be recovered as free choline. No detectable choline was released into the perfusate during the initial 30 min of equilibration. There was no decrease in choline concentration after removal of the liver from the apparatus.

During the first 15 min after addition of labeled substrate to the perfusate, the uptake of choline was approximately linear. The concentrations of choline at midpoints of these initial periods and the slopes of the corresponding linear approximations were used as data points in Fig. 1, which shows the initial velocity of choline uptake as a function of substrate concentration in the perfusate. The uptake of choline did not follow strict Michaelis–Menten kinetics; no maximum velocity was reached in the range of concentrations studied. However, the uptake became linear when choline concentrations were greater than 0.3 mM. We assumed that the observed net uptake was the result of two processes, one saturable and obeying Michaelis–Menten kinetics and the other nonsaturable and linear. The hyperbola was characterized statistically by two methods, both of which yielded similar Michaelis–Menten constants.

(*i*) The first method was the use of repetitive iterations of the difference between observed uptake and the linear component by using direct hyperbolic regression fitting with weighting of 1/variance as described by Wilkinson (23). By this method the saturable component had a $K_a = 0.13$ mM ± 0.05 (SEM) for choline and a $V_{max} = 0.69$ μmol/min × g dry weight ± 0.05 (SEM). The nonsaturable component had a slope of 0.26 μmol/min × g dry weight (calculated by linear regression, $r = 0.8960$; $P < 0.01$).

(*ii*) The second method was the use of nonlinear regression analysis (24) with the equation

$$V = (S \times V_{max})/(S + K_m) + mS + b,$$

in which V = velocity of choline uptake (μmol/min × g dry weight) and S = substrate concentration (mM). By this approach $K_a = 0.17$ mM ± 0.07 (SD) for choline and $V_{max} = 0.84$ μmol/min × g dry weight ± 0.16 (SD). The nonsaturable component had a slope of 0.30 ± 0.04 (SD) μmol/min × g dry weight. Because residual errors for all data points were of the same magnitude, no weighting was used. This method of calculation involves only one regression; thus it avoids the compounding of errors that occurs in method *i*. By either method, similar K_m and V_{max} values are derived.

After 90 min of perfusion with media containing low initial concentrations of choline (0.02–0.125 mM), 90% of the radioactivity within the liver was accounted for by choline, betaine, phosphorylcholine, and lecithin (Fig. 2*A*). After perfusions with high initial concentrations of choline (2.5–4.5 mM), the radioactivity was found in the same metabolites, though relatively more was found in betaine (Fig. 2*B*).

In five open-circuit perfusion experiments, intracellular accumulation of choline-derived label was calculated by inulin determination of extracellular space. Liver uptake of choline calculated by this method agreed well with uptake calculated by choline's disappearance from perfusate (see Fig. 1).

In three *in situ* perfusion experiments, accumulation of intracellular free choline was calculated by using the inulin space [0.15 ml ± 0.004 (SD)/g dry weight] and assuming a constant specific activity of the infused choline. No radioactive lecithin was detected in liver after the 1-min infusion; virtually all of the intracellular label was identifiable as choline, betaine, or phosphorylcholine. Between 40 and 124 nmol of labeled free choline per g dry weight accumulated within hepatocytes.

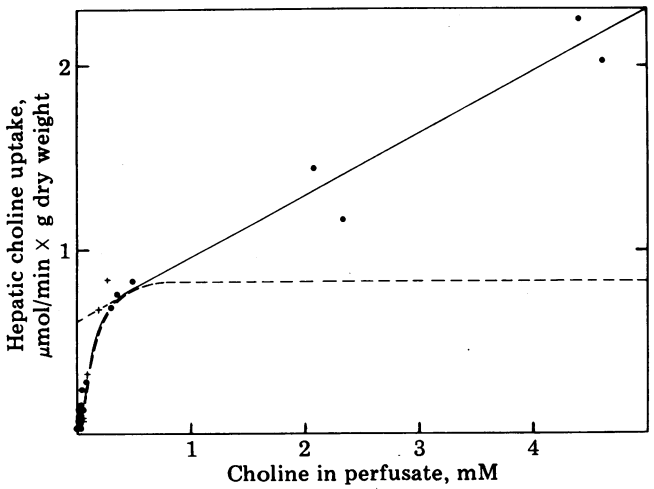

FIG. 1. Uptake of choline by perfused rat liver. ●, Uptake rates calculated from choline disappearance in closed-circuit perfusions; +, rates of choline uptake calculated by using accumulation of intracellular isotopically labeled choline in open-circuit perfusions of isolated and *in situ* livers. —, Constructed by using nonlinear regression analysis; - - -, the saturable component of the uptake.

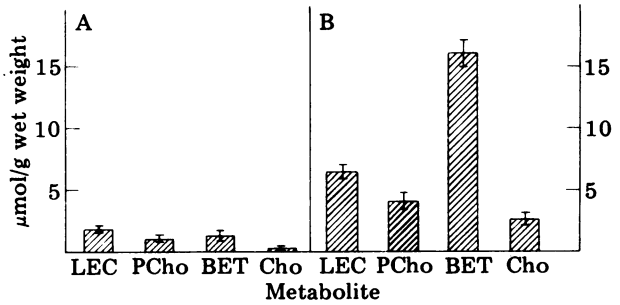

FIG. 2. Metabolites formed from choline in isolated perfused rat liver. The metabolites were assayed in livers perfused for 90 min with radioactive choline. Perfusate choline concentrations were 0.005–0.125 mM (*A*) and 2.5–4.5 mM (*B*). Data are expressed as μmol of choline-derived label per g wet weight ± SEM. LEC, lecithin; PCho, phosphorylcholine; BET, betaine; Cho, choline.

DISCUSSION

These studies demonstrate that the liver rapidly removes choline from the portal circulation. It appears that net uptake is the sum of at least two processes, one which takes up choline at a rate proportional to its concentration in the perfusate and one which exhibits saturable enzyme kinetics. Both mechanisms are unsaturated at physiological concentrations of choline in plasma (0.01–0.02 mM) and at concentrations of plasma choline reached when rats or humans are treated with choline-containing compounds (0.03–0.08 mM) (3–5). Postprandial portal venous choline concentrations might exceed the K_m of the saturable mechanism if all absorbed free choline enters the portal circulation. We found that the maximum velocity of this mechanism should be sufficient to allow it to clear plasma of choline rapidly. Our results are consistent with the rates of hepatic choline uptake reported by Tuma *et al.* (14–16). From their data (15) one can calculate uptakes of 0.80 and 1.33 μmol/min × g dry weight at initial perfusate choline concentrations of 1.25 and 2.50 mM, respectively. From data collected *in vivo* in guinea pigs (6) one can calculate the rate of choline disappearance from plasma to be approximately 0.25 μmol/min × g dry weight of liver over a concentration range of 0.03–1 mM. This is comparable with the rates we observed in perfused rat liver.

The isotopically labeled products found within liver can be formed by using known pathways of choline metabolism. At lower choline concentrations (0.005–0.125 mM), phosphorylcholine and lecithin (synthesized from phosphorylcholine; ref 25) account for nearly 70% of the metabolites of choline and betaine accounts for approximately 60% of labeled metabolites, phosphorylcholine plus lecithin account for 30%, and choline accounts for 10%. This may indicate that the enzyme that converts choline to phosphorylcholine (choline kinase) is saturated at lower choline concentrations than is the enzyme system responsible for synthesizing betaine [choline oxidase, which is composed of choline dehydrogenase (EC 1.1.99.1) plus betaine-aldehyde dehydrogenase (EC 1.2.1.8)]. This agrees with the observed K_m values for choline of these enzymes—namely, 0.33 mM and 0.7 mM for choline kinase (26) and choline oxidase (27), respectively.

It has been suggested that choline uptake into the liver is directly linked to choline oxidase activity (14). If the degradation of choline were rate limiting for choline's uptake into the cell, then no free choline should ever accumulate in liver. We found that labeled free choline does accumulate in amounts that compare in magnitude to those previously reported for total free choline in liver (50–350 nmol/g) (28, 29). It may be that free choline accumulates only at high perfusing choline concentrations, when the linear uptake mechanism makes a significant contribution to the process. Thus the possibility remains that the saturable choline uptake mechanism is in some way linked to oxidase activity.

The liver's ample ability to act as a sink for choline can explain the rapid clearance of administered choline from plasma. Oral administration of 2.3 g of choline chloride to humans causes a 3-fold rise in plasma choline concentration (from 0.01 to 0.03 mM). Assuming that choline is distributed in the extracellular fluid, the observed increase in plasma choline would represent less than 5% of the administered dose. It might be expected that when liver function is impaired (e.g., in cirrhosis), plasma choline concentrations might be reached with small doses if hepatic choline uptake would be pharmacologically inhibited.

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