Uptake of choline by rat mammary-gland epithelial cells

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The neonatal mammal requires especially large amounts of choline to sustain growth. Much of this choline is derived from the newborn's only source of food, milk. The concentration of choline in rat milk [$182 \pm 24 \mu\text{M}$ (S.E.M.)] was much higher than that in maternal serum ($11.6 \pm 0.9 \mu\text{M}$), suggesting that a mechanism capable of concentrating choline into milk must exist. We characterized choline uptake by mammary epithelial cells (the site of milk production) of the lactating rat. We observed two uptake processes, one saturable and obeying Michaelis–Menten kinetics, and the other non-saturable and linear. At physiological blood choline concentrations, the saturable component of choline uptake predominated. The saturable component had $K_{\text{app.}} = 35 \pm 16 \mu\text{M}$, and $V_{\text{max.}} = 1.24 \pm 0.19 \text{ nmol/h}$ per mg of protein. Saturable uptake of choline was inhibited by hemicholinium-3. Ca^{2+} was required for uptake, but Mg^{2+} was not. Replacement Na^+ with K^+ , Li^+ or sucrose inhibited transport. Ouabain did not inhibit choline uptake. Choline concentration in epithelial cells was $67.7 \pm 1.9 \text{ nmol/g}$ wet wt. at the start of incubation at 37°C and rose to $80.9 \pm 6.5 \text{ nmol/g}$ wet wt. over 30 min. Much of the choline accumulated by the mammary gland (in the presence of endogenous concentrations of choline) remained in the form of choline ($50 \pm 1.2\%$), phosphatidylcholine ($12 \pm 2.3\%$), lysophosphatidylcholine ($0.1 \pm 0.03\%$), betaine ($7 \pm 0.3\%$) and phosphocholine ($6 \pm 0.5\%$). In addition, we isolated $25 \pm 1.2\%$ of choline-derived radiolabel in an unidentified compound.

INTRODUCTION

Choline is an essential nutrient for many mammals [1]. It is a precursor for the biosynthesis of phosphatidylcholine, sphingomyelin and choline plasmalogens, which are all essential constituents of membranes. It is a major source for methyl groups, and is also needed to make acetylcholine, an important neurotransmitter [1]. For many mammals, ingestion of a diet deficient in choline has major consequences, including fatty infiltration of the liver, renal dysfunction, and increased sensitivity to carcinogens [1].

The neonatal mammal requires especially large amounts of choline to sustain growth as well as for normal maintenance of tissue mass [1–3]. Much of the choline needed by the newborn is derived from its only source of food, milk, which contains high concentrations of this amine [2,4]. In humans, unesterified choline concentrations in milk are highest ($> 600 \mu\text{M}$) during the first week of lactation, and decreased, thereafter, to $100\text{--}200 \mu\text{M}$ [4]. Human milk also contains choline in the form of phosphatidylcholine ($100\text{--}200 \mu\text{M}$) and sphingomyelin ($100\text{--}200 \mu\text{M}$) [4].

The choline in milk could be transported there from maternal blood. Milk choline concentration is $10\text{--}20$ -fold higher than that present in maternal serum [5]. In order for transport to occur, an uptake mechanism would have to exist within the mammary epithelial cell (the site of milk production) which is capable of concentrative transport of choline. The uptake of choline has been investigated in a variety of tissues such as cholinergic neurons, diaphragm, liver, erythrocytes,

kidney, placenta and intestine [6–16]. We now characterize choline uptake by isolated mammary epithelial cells from the lactating rat.

METHODS

Animals

Lactating female Sprague–Dawley rats (Charles River Breeding Laboratories, Wilmington, MA, U.S.A.) were housed in polyethylene tubs with their litters (nine or ten pups). They were offered food (Rodent Lab Chow, Farmers Exchange, Framingham, MA, U.S.A.) and water *ad libitum*. On day 15 *post partum*, dams were decapitated, and mammary tissue was collected.

Isolation of epithelial cells

Mammary epithelial cells were isolated and enriched by the method of Richards *et al.* [17]. Mammary glands were freed of fascia and minced into 0.4 mm pieces on a chilled work surface. These were suspended in isolation buffer [Krebs–Henseleit bicarbonate buffer (100 ml of 0.9% NaCl , 4 ml of 1.15% KCl , 3 ml of 1.22% CaCl_2 , 1 ml of 2.11% KH_2PO_4 , 1 ml of 3.8% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.3% NaHCO_3 , gassed with CO_2 to $\text{pH } 7.4$), $\text{pH } 7.4$, containing 4% (w/v) bovine serum albumin and 10 mM glucose] and collagenase (10 mg/g of tissue; type III; Worthington Biochemicals, Freehold, NJ, U.S.A.) was added. Tissue was incubated for 1.5 h at 37°C , with gentle mixing every 30 min. The mixture was centrifuged at $500 g$ for 10 min at 4°C , and the supernatant was aspirated (removing adipocytes). The pellet was

Abbreviations used: PNA, peanut lectin.

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resuspended in isolation buffer and filtered through a nylon mesh (400 μm pore) to remove undigested tissue. The cells which passed through the screen were washed three times with isolation buffer and were resuspended at 1.5×10^7 cells/ml in isolation buffer. Deoxyribonuclease (50 $\mu\text{g}/\text{ml}$; type I; Sigma Chemical Co., St. Louis, MO, U.S.A.) was added to prevent cell clumping. This tissue digest was further enriched for epithelial cells by isopycnic banding on a density gradient. The density gradient was prepared in a 50 ml polycarbonate tube containing 10.8 ml of Percoll (Pharmacia, Piscataway, NJ, U.S.A.), 2.8 ml of 9% (w/v) NaCl and 14.4 ml of water. A sigmoid gradient was formed after centrifugation in a fixed-angle rotor at 20000 g for 60 min at 4 °C. The cells were layered on the gradient, and centrifuged in a swinging-bucket rotor at 800 g for 15 min at 4 °C. Epithelial cells banded at a density (1.05–1.07 g/ml) below most stromal cells and cell debris, and above nuclei and erythrocytes. The epithelial band was collected and diluted in 7 vol. of isolation buffer, then centrifuged at 100 g for 5 min at 4 °C, and the pellet was used in uptake studies.

Cell survival was assessed by using Trypan Blue exclusion; in all experiments viability exceeded 80–95%. Our mammary-cell preparation accumulated 3-*O*-methyl[^3H]glucose at rates that were similar to those reported by other investigators [18]. We used immunohistochemical methods to evaluate the purity of epithelial cells [19,20]. Cells were delivered on to microscope slides by cytocentrifugation, and were fixed in acetone. These were incubated with peanut lectin (PNA; Vector Laboratories, Burlingame, CA, U.S.A.) which specifically binds to a galactose disaccharide marker on the surface of the mammary epithelial cell. The cell-PNA complex was then incubated with rabbit anti-PNA antibodies (DAKO Corp., Santa Barbara, CA, U.S.A.) and with swine anti-(rabbit Ig) antibody (DAKO Corp.). Horseradish peroxidase–rabbit anti-(horseradish peroxidase) immune complexes (PAP; DAKO Corp.) were added, and bound to the swine anti-(rabbit Ig) antibody. Slides were washed, and H_2O_2 was added as a substrate for the peroxidase. 3,3'-Diaminobenzidine tetrahydrochloride (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) was used as a chromogen. Cells were counter-stained with haematoxylin; 99% of our isolated cells stained positively as epithelial cells. When appropriate controls were used (no PNA, or PNA with galactose added to prevent binding), cells did not stain. The cells had characteristic features of mammary epithelial cells when examined with electron microscopy (results not shown).

Measurement of choline uptake by mammary gland *in vivo*

Choline chloride (100 mg/kg; Sigma) was administered via orogastric intubation to lactating rats (10 days *post partum*). Blood was obtained by intracardiac puncture under light diethyl ether anaesthesia. Milk was obtained by manual expression after intraperitoneal injection of oxytocin (0.2 ml of Syntocin, 10 units/ml; Sandoz Pharmaceuticals, East Hanover, NJ, U.S.A.). Choline was measured in milk and serum.

Measurement of choline uptake *in vitro*

Epithelial cells were suspended in Krebs–Henseleit bicarbonate buffer, pH 7.4, containing 10 mM-glucose

(200 mg of cells/ml). Cell suspension (100–200 μg of protein) was delivered to wells of a tissue-culture plate (Falcon; Fisher Scientific, Medford, MA, U.S.A.). Various amounts of choline chloride and 1 μCi of [*methy*l- ^3H]choline chloride (80 Ci/mmol; New England Nuclear, Boston, MA, U.S.A.) were added as indicated. Cells were incubated for 30 min at 37 °C, and then were collected on filter paper (Filtermats; Skatron, Sterling, VA, U.S.A.) by using a semi-automatic cell harvester (Skatron). The wash volume per well was 5 ml. Cells on filter paper were placed in scintillation vials; 500 μl of methanol was added, followed by scintillation fluid (Scintiverse E; Fisher Scientific). Radioactivity (c.p.m.) was determined by scintillation spectrophotometry (LKB Rackbeta) and d.p.m. were calculated by using the external-standard channels ratio. In all uptake studies, a blank to correct for [^3H]choline in the extracellular water space and for non-specific adsorption of choline. We used a 30 min incubation period when we studied uptake for all reported studies. In pilot experiments, we demonstrated that uptake proceeded linearly with time between 5 and 60 min of incubation.

As indicated other chemicals (ouabain, 2,4-dinitrophenol, hemicholinium-3, LiCl, KCl, sucrose; Sigma) were added to incubation mixture before addition of radiolabelled choline. In some experiments, as indicated in the Figure legends, the Krebs–Henseleit bicarbonate buffer was prepared without Ca^{2+} , or without Mg^{2+} or without Na^+ .

Identification of radiolabelled choline metabolites

Selected cell pellets were kept after incubation with [^3H]choline, and were extracted by the method of Bligh & Dyer [21]. The aqueous phase was kept and used for determination of aqueous metabolites of choline. Samples of the organic extract were applied to silica-gel plates (Si50PA; Baker Chemical, Phillipsburg, NJ, U.S.A.). These were developed with chloroform/methanol/water (65:30:4, by vol.). The bands which co-chromatographed with authentic phosphatidylcholine (99% pure dipalmitoyl; Sigma), lysophosphatidylcholine (99% pure palmitoyl; Sigma) or sphingomyelin (99% pure dipalmitoyl; Sigma) standard were identified with iodine vapour, and were scraped off the plates and transferred to a scintillation vial, dried under N_2 , resuspended in 500 μl of methanol and 5 ml of scintillation fluid (Scintiverse E), and radioactivity (c.p.m.) was determined by liquid-scintillation spectrophotometry.

A sample of the aqueous phase of tissue extracts was used to determine the distribution of radiolabel among metabolites of choline. We used a modification of the method of Liscovitch *et al.* [22]. Dried sample was resuspended in methanol/water (2:1, v/v) and was applied to a silica h.p.l.c. column (Pecosphere-3CSi, 5 μm particle size; 4.6 mm $\times$ 83 mm; Perkin–Elmer). Metabolites were eluted with a non-linear gradient of acetonitrile/ethanol/acetic acid/1 M-ammonium acetate/100 mM-sodium phosphate/water (800:68:2:3:10:127, by vol., changing to 200:34:22:44:5:200, by vol.). Peaks were detected with an on-line radioactivity monitor (LB 506A with solid flow cell; Berthold, Nashua, NH, U.S.A.). Fractions were collected corresponding to the peaks of radioactivity.

Protein assay

Protein was measured using the colorimetric assay of Bradford [23].

Choline assay

The cell pellet was extracted with 1 M-formic acid/acetone (3:17, v/v) and choline was isolated from the resulting supernatant by ion-pair extraction with dipicrylamine into dichloromethane [24]. Choline was then converted into the propionyl ester, and was demethylated with sodium benzenethiolate. This volatile derivative was then isolated by g.l.c. (1.83 m × 2 mm internal diam. glass column packed with 3% OV-17 on GC22 precoated with 1% 4-dodecyldiethylenetriamine succinamide) and measured with a mass-selective detector (Hewlett-Packard 5970 GC/MSD) [25]. An internal standard of deuterated choline was used to correct for variation in recovery.

RESULTS

Effect of treatment with choline upon milk and serum choline concentrations

Choline concentration in serum was $11.6 \pm 0.9 \mu\text{M}$ (S.E.M.) whereas in rat milk it was $182 \pm 24 \mu\text{M}$. When lactating rats were treated with choline, serum choline concentration increased 2-fold over 6 h ($P < 0.01$ by one-way ANOVA and Scheffe's test [26]; Fig. 1). During the same period, milk choline concentration increased more than 8-fold ($P < 0.01$ by one-way ANOVA and Scheffe's test; Fig. 1).

Choline concentrations in mammary cells

Choline concentration in mammary cells was $67.7 \pm 1.9 \text{ nmol/g wet wt.}$ at the start of incubations for measurement of choline uptake. After 30 min of incubation at 37°C with no added choline, the

Fig. 1. Choline administration increases choline concentration in serum and milk of the rat *in vivo*

Choline chloride (100 mg/kg; Sigma) was administered via orogastric intubation to lactating rats (10 days *post partum*). Blood was obtained, at the times indicated, by intracardiac puncture under light ether anaesthesia. Milk was obtained, at the times indicated, by manual expression after injection of oxytocin. Choline was measured in milk and serum as described in the Methods section. Data are expressed as mean choline concentrations $\pm$ S.E.M. ($n = 5$ per point). * $P < 0.05$, ** $P < 0.01$ by Student's t test.

concentration of this amine within cells was $80.9 \pm 6.5 \text{ nmol/g wet wt.}$ Choline concentrations in the supernatant at the start and end of such an incubation were 10.6 ± 0.4 and $26.3 \pm 0.5 \text{ nmol/ml}$ respectively.

Calculation of kinetic constants

The uptake of choline-derived radiolabel by mammary cells did not follow strict Michaelis-Menten kinetics (Fig. 2); no maximal velocity was reached in the range of choline concentrations studied. The uptake became linear when choline concentrations were greater than $200 \mu\text{M}$. We assumed that the observed net uptake was the result of two processes, one saturable and obeying Michaelis-Menten kinetics, and the other non-saturable and linear (Fig. 2). We used non-linear regression analysis (Fitfunction on a Digital VAX computer; BBN Research Systems, Cambridge MA, U.S.A.) with the equation:

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

in which v = velocity of choline uptake, S = choline concentration and m = slope of non-saturable component. The best fit ($r^2 = 0.9894$, $F = 249$, $P < 0.0001$) estimates for the kinetic constants of the saturable component were: $K_{\text{app}} = 35 \pm 16 \mu\text{M}$, $V_{\max, \text{app}} = 1.24 \pm 0.19 \text{ nmol/h per mg of protein}$. The non-saturable component had a slope (m) of $0.6 \text{ (mg of protein} \cdot \text{h}^{-1} \cdot \text{l}^{-1})$. The regression fit was not as good ($r^2 = 0.9133$) when we assumed that the data could be described by a simple hyperbola.

Fig. 2. Choline uptake by rat mammary cells

Mammary epithelial cells were suspended in Krebs-Henseleit bicarbonate buffer, pH 7.4, containing 10 mM-glucose. [*methyl-³H*]Choline chloride was added at the concentrations indicated. Cells were incubated for 30 min at 37°C , and then were collected on filter paper. Radioactivity (d.p.m.) was determined by liquid-scintillation spectrophotometry. In all uptake studies, cells which were not incubated at 37°C were used as a blank. Data are expressed as mean choline uptake $\pm$ S.E.M. ($n = 3-10$ per point). We used non-linear regression analysis with the equation $V = [(S \times V_{\max}) / (S + K_m)] + mS$ to construct a curve fitting the data (—; $r^2 = 0.9894$). The linear (---) and hyperbolic (----) components of choline transport are indicated separately.

Effects of potential inhibitors of choline transport

Hemicholinium-3 (1 mM), an analogue of choline, inhibited choline uptake. Inhibition was greatest when choline concentration in the medium was low (60% inhibition; $P < 0.01$ different from control by Student's t test; Fig. 3). This inhibition was overcome by the addition of more choline to the medium. We were never able to inhibit choline transport completely with hemicholinium-3; we calculated that at 100 μM -choline the saturable uptake process accounted for 94% of observed transport. Hemicholinium-3 inhibited by 74%. Ouabain (20 μM or 150 μM), an inhibitor of the Na^+/K^+ -pump ATPase, did not inhibit choline uptake, but enhanced it by approx. 50% ($P < 0.05$ at all choline concentrations by Student's t test; Fig. 3). Ouabain at higher concentration (1 mM) did not affect choline transport ($96 \pm 0.7\%$ of control). When oxidative phosphorylation and/or electron transport was uncoupled with 2,4-dinitrophenol (1 mM), choline uptake was inhibited by 25–40% ($P < 0.05$ by Student's t test; Fig. 3).

Effect of removing glucose

Choline transport was not inhibited by the omission of glucose from the incubation medium. At 0, 100 and 800 μM added choline, we observed $116 \pm 18\%$, $156 \pm 28\%$ ($P < 0.05$ for difference from control by Student's t test) and $132 \pm 46\%$ as much choline transport, respectively, as in controls.

Effects of altered ionic environment

Replacement of Na^+ in the incubation medium with osmotically equivalent amounts of Li^+ , K^+ or sucrose inhibited choline uptake by 40–65% ($P < 0.05$ for sucrose, $P < 0.01$ for Li^+ and K^+ for difference from control by Student's t test; Fig. 4). Total inhibition of the

Fig. 3. Effects of potential inhibitors of choline transport

Mammary cells were incubated as described for Fig. 2, except that hemicholinium-3 ($\square$; 1 mM), 2,4-dinitrophenol ($\boxtimes$; 1 mM) or ouabain ($\boxdot$; 150 μM) was added at the start of incubation. Data are expressed as percentages of control choline uptake as means $\pm$ S.E.M. ($n = 3$ –5 per point): * $P < 0.05$, ** $P < 0.01$ by Student's t test.

Fig. 4. Effects of altered ionic environment

Mammary cells were incubated, with added 100 μM -choline, as described for Fig. 2. In some samples iso-osmotic amounts of sucrose (Sucrose-no Na), LiCl (Li-no Na) or K^+ (K-no Na) were used instead of Na^+ in the incubation buffer. In other samples either Ca^{2+} (no Ca) or Mg^{2+} (no Mg) was omitted from the buffer. Data are expressed as percentages of control as mean $\pm$ S.E.M. ($n = 3$ –5 per point): * $P < 0.05$, ** $P < 0.01$ by Student's t test.

saturable component of choline uptake would have blocked 95% of transport. Omission of Ca^{2+} from the incubation medium inhibited uptake by 84% ($P < 0.01$ for difference from control by Student's t test; Fig. 4). Omission of Mg^{2+} from the incubation medium enhanced uptake by 60% ($P < 0.01$ for difference from control by Student's t test; Fig. 4).

Metabolism of choline within mammary cells

Much of the choline-derived radiolabel accumulated by mammary cells during a 30 min incubation remained in the form of choline (Table 1). Radiolabel was also recovered in phosphatidylcholine, betaine and phosphocholine, as well as in an unknown water-soluble compound (Table 1). This unknown compound was not methionine, acetylcholine, glycerophosphocholine, S-adenosylmethionine, trimethylamine or dimethylamine (as determined by different elution times in our h.p.l.c. system). It could not be cleaved to form free choline by incubation in 6 M-HCl at 130 $^{\circ}\text{C}$ for 1 h or in 1.2 M- NH_3 at 37 $^{\circ}\text{C}$ for 30 min.

DISCUSSION

Unesterified choline concentrations were always much higher (more than 15-fold; Fig. 1) in milk than in serum obtained simultaneously. At 6 h after oral administration of choline, there was more than 60-fold higher choline concentration in milk than in serum. For choline to be taken up from serum and concentrated in milk, the mammary epithelial cell must possess a mechanism for mediated transport of choline.

We found that mammary epithelial cells accumulated choline-derived radiolabel. Most of the radiolabel remained in the form of choline or choline esters after incubation, therefore we are confident that we have characterized the movement of choline molecules into the epithelial cell. Hemicholinium-3 has been widely used in experiments which have examined choline uptake [6–9]. It is a specific competitive inhibitor of choline

Table 1. Metabolites of choline formed by mammary cells

Selected mammary cell pellets were extracted after incubation with [^3H]choline as described in the Methods section. Samples of the organic extract were chromatographed on silica-gel t.l.c. plates developed with chloroform/methanol/water. Radioactivity (d.p.m.) in the bands which co-chromatographed with authentic standards was determined by scintillation spectrophotometry. A sample of the aqueous phase of tissue extracts was chromatographed by h.p.l.c. Peaks were detected with an on-line radioactivity monitor. Retention times are indicated in min. The unknown aqueous soluble compound was not methionine, acetylcholine, glycerophosphocholine, *S*-adenosylmethionine, trimethylamine or dimethylamine (as determined by different elution times in our h.p.l.c. system). It could not be cleaved to form free choline by incubation in 6 M-HCl at 130 °C for 1 h, or in 1.2 M-NH₃ at 37 °C for 30 min. Results are means $\pm$ S.E.M. ($n = 4$ per choline concentration): nd, not detected.

Metabolite	H.p.l.c. retention time (min)	Radiolabel accumulated by cell (% of total)	
		No added choline	400 μM -choline
Choline	11.1	50.1 $\pm$ 1.2	72.2 $\pm$ 1.4
Betaine	4.3	7.3 $\pm$ 0.3	3.2 $\pm$ 0.4
Phosphocholine	19.0	6.1 $\pm$ 0.5	2.9 $\pm$ 0.3
Unknown	0.8	24.5 $\pm$ 1.2	15.7 $\pm$ 1.3
Glycerophosphocholine	15	nd	nd
CDP-choline	17	nd	nd
Acetylcholine	9.1	nd	nd
Lysophosphatidylcholine	—	0.1 $\pm$ 0.002	nd
Phosphatidylcholine	—	12.0 $\pm$ 2.3	5.9 $\pm$ 0.2
Sphingomyelin	—	nd	nd

transport carrier systems. Choline uptake by mammary epithelial cells was inhibited by hemicholinium-3, suggesting that we are characterizing a carrier specific for choline, rather than some non-specific leak.

Uptake of choline by mammary cells was best described as the sum of a saturable and a non-saturable process (Fig. 2). Such a combination of choline transport processes in a single cell is not unusual, and has been described in brain, liver, erythrocytes, placenta and intestine [6,8–12,16]. We suggest that the non-saturable process that we observed in mammary cells was probably diffusion, as it was most important at high choline concentrations (i.e. when choline in the medium exceeded intracellular choline concentration). Saturable uptake predominated at choline concentrations below 100 μM (when intracellular choline concentrations were higher than those of the medium). Thus it is likely that this mechanism involved facilitated transport. Choline uptake proceeded linearly with time (between 5 and 60 min), suggesting active transport. Choline transport was inhibited by 2,4-dinitrophenol, (Fig. 3), suggesting that oxidative phosphorylation is required. Serum choline concentration in the rat or human is normally approx. 10 μM , and exceeds 50 μM only after pharmacological doses of choline or choline-containing compounds have been administered [27,28]. Concentrations as high as 100 μM have never been reported. Therefore, under physiological conditions it is likely that the saturable mechanism that we have described is the important transporter of choline in the mammary.

Marked changes in choline uptake by mammary cells occurred when we perturbed the ionic milieu of the cells. Omission of Mg²⁺ from the medium enhanced choline uptake (Fig. 4). We have no definitive explanation for this phenomenon, but the data make it clear that a Mg²⁺-dependent enzyme was not required for transport. Our data suggest that choline transport was Ca²⁺-dependent (Fig. 4), as omission of Ca²⁺ from the medium markedly inhibited transport. Choline uptake into the

myenteric plexus of the intestine also has been shown to require Ca²⁺, but the high-affinity choline transporter in brain neurons does not [13,14]. Complete omission of Na⁺ and replacement by Li⁺, K⁺ or sucrose consistently inhibited choline uptake by mammary cells (Fig. 4). Li⁺ by itself can irreversibly inhibit certain choline-transporting systems, via an unknown mechanism [15]. Since high K⁺ is commonly used to depolarize cells, it is possible that under physiological conditions K⁺ concentration differences provide the electrochemical gradient that is the driving force for choline accumulation. High K⁺ decreases choline uptake by placenta [6], erythrocyte [8] and brain tissue [16]. In brain, choline uptake is inhibited by ouabain [13,16], but in erythrocytes it is not [8]. At low ouabain concentrations ($\leq 150 \mu\text{M}$), choline transport was stimulated (Fig. 3). In cardiac muscle the existence of two specific ouabain receptors has been postulated: a high-affinity site related to the Na⁺–K⁺ pump [Mg²⁺-dependent Na⁺- and K⁺-activated ouabain-sensitive ATPase (EC 3.6.1.3)] stimulation and a low-affinity site associated with Na⁺–K⁺-pump inhibition [29]. Perhaps the high-affinity site is also present in mammary tissue, and influences choline accumulation. We were not able to demonstrate inhibition of choline uptake at high ouabain concentrations. This discounts the possibility that a K⁺-dependent electrochemical gradient is the driving force for choline accumulation as this enzyme helps to maintain the high intracellular concentration of K⁺. Na⁺-dependence of the mammary choline transporter would explain the effects of Li⁺, K⁺ and sucrose that we observed. The choline transporter in brain [13,16], kidney [7], and erythrocyte [8] has a requirement for Na⁺.

Mammary tissue used a small amount of the accumulated choline to form betaine, thus choline dehydrogenase (EC 1.1.99.1) and betaine-aldehyde dehydrogenase (EC 1.2.1.8) must be present in mammary cells. This enzyme activity has not previously been

reported in mammary gland. Phosphocholine and phosphatidylcholine were also formed, probably as products of the Kennedy [30] pathway. The unknown aqueous metabolite of choline made by mammary tissue is extremely interesting, as in our experience it is unique to mammary tissue. We have incubated choline with liver and brain, and have never observed formation of this unknown metabolite. It was not a choline ester, as we could not liberate free choline by acidic or basic cleavage. It is possible that it is a metabolite of betaine, such as dimethylglycine or sarcosine.

In the studies we describe above, we characterized net transport of radiolabelled choline. We also found that choline concentrations increased within mammary cells even when no choline was added to the incubation medium. This occurred because mammary gland is capable of biosynthesis *de novo* of choline molecules via the sequential methylation of phosphatidylethanolamine to form phosphatidylcholine [31].

In summary, we have identified a mechanism in mammary cells which is capable of concentrative uptake of choline. We suggest that this mechanism, along with synthesis of choline *de novo* catalysed by phosphatidylethanolamine *N*-methyltransferase [31], makes large amounts of choline available within the mammary cell where milk is made. This would explain why milk has choline concentrations that are 10–20-fold higher than in maternal blood. It would be interesting to determine whether factors known to influence milk production (e.g. hormones, diet) also affect mammary uptake of choline.

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