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Biochemistry

Synthesis of choline from ethanolamine in rat brain

([*methyl*-^{14}C]methionine/lecithin/acetylcholine)

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ABSTRACT **Specific radioactivities of choline, acetylcholine, phosphocholine, lecithin, lysolecithin, and glycerophosphorylcholine have been measured in brain, blood, liver, and muscle after the intravenous injection of three labeled precursors: choline, methyl-labeled methionine, and ethanolamine.**

In relation to the specific activity of free choline in blood there was significantly more radioactivity in the free choline of brain after administration of methyl-labeled methionine and labeled ethanolamine than after labeled choline. Since the choline moiety of lipids, which returns back to the choline pool, contained less radioactivity after methyl-labeled methionine and labeled ethanolamine than after labeled choline, it is the most likely interpretation of the finding that choline, in brain can be formed by methylation of free ethanolamine.

Data from liver confirm that lecithin is formed in the liver by methylation of phosphatidylethanolamine.

No indication was found for the synthesis of choline in muscle. Rates of transfer and transport of choline in brain have been calculated as nmol $\times$ g^{-1} $\times$ min^{-1} as follows: turnover rate of choline, 36.5; rate of synthesis of choline by methylation and net loss of choline into the bloodstream, 6.3; inflow from the blood, 6.2; outflow into the blood, 12.5; transfer into lipids and vice versa, 20; transfer to acetylcholine and vice versa, 4.

It has been suggested that choline (Ch) might be synthesized in rat brain by methylation of free ethanolamine (EA) (1, 2). To give direct evidence of this, experiments have now been performed with three labeled compounds likely to be precursors of Ch in brain, namely Ch, methyl-labeled methionine, and EA. If Ch is not only delivered to the brain via the bloodstream but also is formed in the brain by methylation of EA, then radioactivity in the free Ch of brain compared to that in blood should be higher after intravenous (i.v.) administration of methyl-labeled methionine or labeled EA than after the administration of labeled Ch. According to previous results, labeled Ch that penetrates from the blood into the brain is there diluted five to six times by unlabeled free Ch formed within the brain. The larger portion of this unlabeled free Ch originates from the breakdown of lipids, namely 20 nmol $\times$ g^{-1} $\times$ min^{-1} (3), but in addition almost 7 nmol $\times$ g^{-1} $\times$ min^{-1} should be synthesized, since the arterial inflow of free choline amounts to 12 but the venous outflow amounts to 19 nmol $\times$ g^{-1} $\times$ min^{-1} (3). If this synthesis of Ch in the brain does occur by methylation of EA, the supply with methyl-labeled methionine or labeled EA would reduce the dilution of the specific radioactivity of the Ch from the blood, which means that the difference between specific radioactivities of free Ch in blood and brain must be smaller.

This would be evidence for methylation of EA in the brain provided that the specific radioactivity of the Ch moiety in the lipids or other choline containing compounds of the brain is not increased considerably.

Abbreviations: Ch, choline; AcCh, acetylcholine; PC, phosphocholine; GPC, *sn*-glycero-3-phosphorylcholine; EA, ethanolamine; MEA, methylaminoethanol; DMEA, dimethylaminoethanol; i.v., intravenous.

MATERIALS AND METHODS

Animals and Chemicals. Male Sprague–Dawley rats (Mus Rattus AG., 8011 Brunnthal, Germany) weighing 125–150 g were used. [*Methyl*-^{14}C]choline chloride, 54.4 mCi/mmol (Amersham, England); [*methyl*-^{3}H]choline chloride, 2.5 Ci/mmol (New England Nuclear); L-[*methyl*-^{14}C]methionine, 56 mCi/mmol (Amersham, England); [*1,2*-^{14}C]ethanolamine hydrochloride, 6.4 mCi/mmol (New England Nuclear); and commercially available chemicals of analytical grade, scintillator grade, or biochemical grade were used.

Labeling Procedure. Each radioactive precursor mixed with appropriate amounts of the two others unlabeled was injected as a single dose into the tail vein. Per 100 g body weight 81.0 $\times$ 10^{6} dpm of [^{14}C]Ch, 76.2 $\times$ 10^{6} dpm of [^{14}C]methionine, or 70.0 $\times$ 10^{6} dpm of [^{14}C]ethanolamine were dissolved in 0.2 ml of 0.9% NaCl solution. After the time indicated (3 or 6 hr) the animals were killed by decapitation.

Extraction of Tissues. All the blood that came out (about 3.0 g), total brain, 2.0 g of each liver, and skeletal muscle from the left hind leg were quickly dissected in this sequence, weighed, and homogenized (Ultraturrax homogenizer, Janke & Kunkel, 7813 Staufen, Germany) in 10 ml (for tissues) or 20 ml (for blood) of ice-cold 95% ethanol containing 0.2% acetic acid according to Stone (1955). The time from decapitation to homogenization was less than 2 min. The blood was collected directly into the ethanol, and thus was denaturated within seconds.

Extraction was equally effective and almost complete for Ch and derivatives, including phosphatidylcholine. After centrifugation an aliquot of the supernatant was dried in a stream of air at 70 °, the dry residue was taken up with 2 ml of 0.5 M acetic acid, and 20 ml of heptane/isoamyl alcohol, 2/1 (vol/vol), were added. After the mixture was shaken for 10 min and the phases had separated the water phase was reextracted with 20 ml of heptane/isoamyl alcohol and then with 20 ml of ether to complete the extraction of the lipids. The water phase was evaporated and the residue was extracted with 2 ml of methanol, of which 1.8 ml was taken for paper chromatography.

Separation of Compounds. This was achieved by paper chromatography in a descending system: Schleicher & Schüll (3354 Dassel, Germany) paper no. 2043 mgl. Solvent mixtures: (*a*) *n*-propanol:0.2 M aqueous ammonium acetate, 4:1; (*b*) *n*-butanol:*n*-propanol:ethanol:formic acid:water, 6:2:1:1:2; (*c*) *n*-butanol:formic acid:water, 8:4:3. Since the conclusions will be based on specific radioactivities (dpm $\times$ nmol^{-1}) and the compounds to be analyzed have similar chemical structures and physico-chemical characteristics, careful separation and purification was of greatest importance.

Several chromatographic steps are necessary to separate Ch and derivatives from EA, methylaminoethanol (MEA), dimethylaminoethanol (DMEA), and derivatives: *Step 1.* Solvent (*a*) and (*b*) consecutively separate mixtures into: fraction 1

Table 1. Specific radioactivities (dpm × $nmol^{-1}$) of choline and derivatives after i.v. administration of radiolabeled choline, methionine, or ethanolamine

	hr	Ch	AcCh	PC	lec	lys	GPC	sph
				Choline				
bl	3	223 ± 11		177, 153	26 ± 3	62 ± 5	27, 22	8 ± 1
	6	137 ± 20		86, 75	41 ± 4	53 ± 9	20, 11	13 ± 2
br	3	33 ± 2	31 ± 1	40 ± 4	3.2 ± 0.2	4.2 ± 0.5	4.5 ± 0.2	0.09 ± 0.01
	6	31 ± 2	19 ± 2	25 ± 3	3.5 ± 0.2	5.1 ± 0.4	6.6 ± 0.4	0.21 ± 0.02
li	3	119, 116		218, 187	169, 73	56, 42	57, 46	12, 12
	6	83, 64		97, 61	81, 57	63, 48	78, 54	35, 25
sm	3	136, 157		ϕ	71, 61	153, 88	134, 51	10, 25
	6	99, 91		ϕ	55, 70	ϕ, 89	160, 112	16, 16
				Methionine				
bl	3	12 ± 0.6		3.3, 1.2	28 ± 1.2	86 ± 5	5.4, 5.4	13 ± 2
	6	22 ± 4		9.1, 7.2	30 ± 0.9	64 ± 6	12, 14	14 ± 8
br	3	16 ± 2	12 ± 0.8	8.3 ± 1.1	0.35 ± 0.04	2.1 ± 0.6	0.7 ± 0.06	ϕ
	6	24 ± 3	13 ± 0.5	11 ± 0.2	0.73 ± 0.03	0.95 ± 0.05	1.4 ± 0.07	ϕ
li	3	ϕ, 41		40, 26	142, 115	70, 69	49, 50	26, 21
	6	45, 42		36,30	84, 132	65, 62	69,60	34, 49
sm	3	17, 11		24, 10	3.2, 1.1	ϕ	6.7, 2.7	ϕ
	6	8.1, 11		15,10	1.2, 1.6	ϕ	6.5, 12	ϕ
				Ethanolamine				
bl	3	1.9 ± 0.8		ϕ	10 ± 0.3	18 ± 2	0.71, 0.71	1.7 ± 0.1
	6	7.5 ± 1.6		5.4, 4.8	24 ± 1	56 ± 1	6.9, 2.3	8.3 ± 1.4
br	3	2.6 ± 0.5	1.2 ± 0.05	1.7 ± 0.2	0.068 ± 0.02	ϕ	0.12 ± 0.04	ϕ
	6	9.8 ± 0.6	8.9 ± 0.5	6.3 ± 0.2	0.28 ± 0.02	ϕ	0.38 ± 0.06	ϕ
li	3	22, 17		5.8, 6.8	50, 40	34, 24	18, 16	7.4, 4.7
	6	33, 34		20, 21	70, 102	57, 58	42, 37	41, 32
sm	3	1.0, 0.96		ϕ	0.58, 0.19	ϕ	ϕ	ϕ
	6	5.1, 4.4		6.1, 2.3	0.46, 0.26	ϕ	ϕ	ϕ

For applied doses and radioactivities see *Materials and Methods*. Values are mean ± SEM if $n > 2$ (4 or 3); when $n = 2$ both values are given. ϕ: impulse rates and/or extinctions too low for reliable specific activities. lec, lecithin; lys, lysolecithin; sph, sphingomyelin; bl, blood; br, brain; li, liver; sm, skeletal muscle.

containing acetylcholine (AcCh), DMEA, and MEA; fraction 2 containing Ch, EA, DMEA, and MEA; fraction 3 containing betaine; fraction 4 containing *sn*-glycero-3-phosphorylcholine (GPC) and phosphocholine (PC), including their EA, MEA, and DMEA analogues. *Step 2.* Solvent (*c*) separates fraction 1 into AcCh, and a mixture of DMEA and MEA; fraction 2 into EA, and fraction 2a containing Ch, MEA, and DMEA. *Step 3.* Solvent (*a*) separates fraction 2a into Ch and a mixture of DMEA and MEA. *Step 4.* Hydrolysis of fraction 4 (100°, 1 M HCl, 1 hr); separation of Ch (from GPC) and PC by steps 2 and 3. *Step 5.* Hydrolysis of PC by acid phosphatase (5); separation of Ch by steps 2 and 3.

The various compounds were localized by cochromatography of authentic samples dissolved in the appropriate organ extracts. Ch and DMEA were stained with Dragendorff's reagent (4), MEA and EA, with ninhydrin. The appropriate spots were cut out and the compounds were eluted. All Ch-containing fractions were further purified by ion pair extraction with dipicrylamine (5).

Phosphatidylcholine, lysophosphatidylcholine, and sphingomyelin were separated by thin-layer chromatography according to Skipski *et al.* (6) and hydrolyzed after elution in a solution of saturated $Ba(OH)_2$, and Ch was isolated by ion pair extraction.

Measurements. AcCh was determined by bioassay using a frog rectus preparation according to MacIntosh and Perry (7). Ch was measured by the dipicrylamine method according to Schill and Danielson (8). Scintillation mixture contained 100 g of naphthalene, 10 g of 2,5-diphenyloxazole (PPO), and 0.25 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (dimethyl-POPOP) per liter of dioxane. A Packard Tri-Carb model 3380 and an absolute activity analyzer model 544 were used.

Rectification of Values. For correction of unavoidable losses of substrates in the analytical procedures a standard amount of ^3H-labeled Ch was added to each sample immediately after homogenization of the tissue and the recovery was calculated. Calculation of specific radioactivity of free choline in brain is based on the average pool size of 27.5 nmol of Ch × g^{-1} (3).

RESULTS

Brain

Three and 6 hr after the injection of labeled Ch the specific radioactivity of Ch in the brain was significantly lower than in the blood (Table 1). No such differences were present after the administration of labeled EA or methionine labeled in the methyl group. This was also clear from the ratios of specific radioactivities of Ch in blood to those in brain (Table 2), which were significantly higher after labeled Ch than after the two other labeled precursors.

For the synthesis of both AcCh and PC the Ch derives directly and exclusively from the pool of free Ch in brain and consequently the specific radioactivities of AcCh and PC were in the same range as those of free Ch in brain no matter from which of the injected precursors the Ch had been labeled.

Due to the much larger pool of lecithin its turnover time is several times the experimental time and therefore the Ch

Table 2. Ratios of specific radioactivities of choline and derivatives in blood and other tissues after injection of labeled precursors

		Choline			Brain		Liver		Muscle	
	hr	bl/br	bl/li	bl/sm	Ch/PC	Ch/lec	Ch/PC	Ch/lec	Ch/PC	Ch/lec
A	3	6.8 ± 0.9	2.1, 1.9	1.8, 1.4	0.8 ± 0.03	10 ± 1	0.6, 0.6	0.7, 1.6	∅	1.9, 2.6
	6	4.4 ± 0.4	1.3, 1.9	1.1, 1.3	1.3 ± 0.2	9 ± 1	0.9, 1.0	1.0, 1.1	∅	1.8, 1.3
B	3	0.67 ± 0.05*	∅, 0.32	0.8, 1.2	2.0 ± 0.2	52 ± 1	∅, 1.6	∅, 0.4	0.7, 1.1	5.2, 10
	6	0.98 ± 0.2†	0.54, 0.73	3.0, 2.8	2.2 ± 0.2	40 ± 4	1.3, 1.4	0.5, 0.3	0.6, 1.0	6.7, 6.7
C	3	0.79 ± 0.4*	0.04, 0.11	0.9, 1.9	1.6 ± 0.2	57 ± 2	3.8, 2.5	0.4, 0.4	∅	1.7, 5.1
	6	0.76 ± 0.1†	0.22, 0.30	1.4, 2.4	1.6 ± 0.1	36 ± 4	1.7, 1.7	0.5, 0.3	0.8, 1.9	11, 17

Various labeled precursors were injected i.v.: (A) [^{14}C]choline, (B) [^{14}C]methionine, (C) [^{14}C]ethanolamine. Values are mean ± SEM if $n > 2$ (3 or 4). Abbreviations are as in Table 1.

* $P < 0.005$ that the ratios are the same as in the experiments with [^{14}C]Ch, as calculated by t-test.

† $P < 0.001$.

moiety of lecithin and also of lysolecithin and GPC contained less radioactivity per mol than free Ch.

In relation to the labeling of Ch, however, specific radioactivities of lecithin were much lower after the administration of labeled methionine and EA than after labeled Ch.

In interpreting these results one must remember that the specific radioactivity of free Ch is highest immediately after the injection and falls over the period of the experiment when labeled Ch is used, whereas after the injection of labeled methionine or EA the Ch in blood and brain is gradually labeled by metabolic processes and the specific activities increase during the experimental period. Thus, the kinetics differ and this should be reflected mostly by differences in the deepest compartment in which the lipids are.

Liver

After injection of labeled Ch the specific radioactivity of Ch in the liver appeared somewhat lower than in blood. The values for the derivatives were all in the same range (Tables 1 and 2). After injection of methyl-labeled methionine or labeled EA, the specific radioactivities of Ch in the liver exceeded those in the blood (Tables 1 and 2). Among all the derivatives lecithin was found to be most highly labeled, which means that lecithin was primarily labeled, as expected from results of Bremer and Greenberg (9), who demonstrated that in the liver phosphatidylethanolamine is methylated to form lecithin.

Also after the administration of labeled Ch the specific radioactivity of lecithin in the liver was rather close to that of Ch, since a large portion of the labeled Ch injected becomes oxidized in the liver to form betaine, from which methyl groups are available for transmethylation. This oxidation of Ch does not occur in brain or muscle.

Muscle

Muscle contains almost half of the total free Ch in the body of rats and represents the largest pool. The ratios of the specific radioactivities of Ch in blood to those in muscle were almost the same, no matter which of the three precursors had been given (Tables 1 and 2). In contrast to liver the highest specific activity was found in the free Ch after each of the precursors, showing that no other compound needs to be considered as labeled primarily.

After the administration of labeled methionine or EA the labeling of lecithin was remarkably higher in muscle than in brain, and it was much lower in muscle (1/10) than in liver.

DISCUSSION

Choline lost from the brain needs to be replaced by new synthesis or by supplementation in the form of Ch derivatives from the bloodstream.

Synthesis of Ch in brain by methylation of EA was first suspected because of the finding of labeled Ch in brain after the i.v. injection of labeled DMEA (1). Supporting evidence came from preliminary results by Kewitz *et al.* (2), which have been expanded and confirmed by the present experiments. The diminution of the difference between the specific radioactivities of Ch in blood and brain after the i.v. injection of methyl-labeled methionine or labeled EA as precursors instead of Ch can hardly be interpreted otherwise.

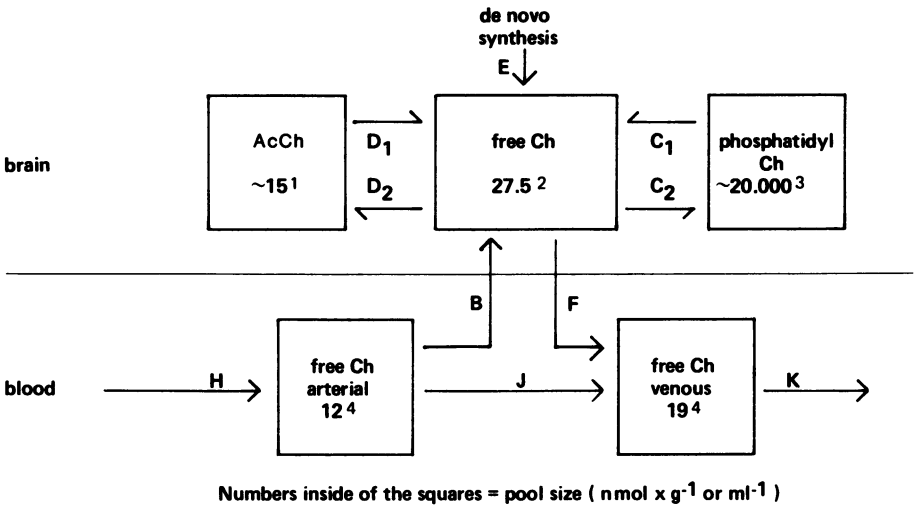
Illingworth and Portman (10), however, demonstrated with doubly labeled lysolecithin in squirrel monkeys that lysolecithin penetrates into the brain from the blood. They suggested this as one of the pathways of Ch supply for the brain. But the data of Illingworth and Portman do not indicate to what extent Ch in brain could be regenerated from lysolecithin.

Our results do not support the assumption of Illingworth and Portman, since specific activities of lysolecithin in brain were much lower than the specific radioactivities of free Ch in brain. Moreover, the total amount of radioactivity in the Ch moiety of lysolecithin in brain after the administration of labeled methionine or EA was less than 50% of that needed for the incorporation into Ch.

In a recent paper Ansell and Spanner (11) mentioned two additional arguments in favor of choline transport to the brain in a lipid-bound form. One is based on their failure to detect significant labeling of choline in blood after the injection of labeled EA or DMEA. This is not in accordance with our results because we found choline in blood considerably labeled. The second argument says that Bjornstad and Bremer (12) were not successful in diminishing adequately the labeling of lipids in various organs by repeated injections of high doses of unlabeled choline following the tracer dose of labeled choline or methionine within 30 min periods. This argument is rather indirect and not conclusive at all and brain was not included in those experiments.

Comparing precursor A (choline) with B (methionine) and C (ethanolamine) in Table 2, the ratios of specific radioactivities of Ch in blood to those in the liver declined with change of compound as in brain. But in the liver the decline was associated with an increase of radioactivity incorporated into the Ch moiety of lecithin.

This can be interpreted as due to methylation at the lipid



Numbers inside of the squares = pool size (nmol x g^{-1} or ml^{-1})

blood flow: 0.9 ml x g^{-1} x min^{-1} [6]

transfer rate	from		to	nmol / g or ml
C_1	free Ch	⇌	phosphatidyl Ch	20[3]
C_2				20
D_1	free Ch	⇌	AcCh	4[5]
D_2				4
E	synthesis	→	free Ch	
	=v.-a. difference x blood flow = 7 x 0.9 =			6.3

transport rate	in				
H	art. blood = concentr. x blood flow = 12 x 0.9 =			10.8	
K	ven. blood = concentr. x blood flow = 19 x 0.9 =			17.1	

calculated on assumption of turnover rates of free Ch in brain (A)

$A = B + C_1 + D_1 + E = F + C_2 + D_2$	=	32.5	36.5	42.5

transfer rate	from		to				
B	blood	⇌	brain	=	2.2	6.2	12.2
F				=	8.5	12.5	18.5

transport rate	in				
J	capillary blood = H − B	=	8.6	4.6	<0

FIG. 1. Rates of transport and transfer of choline in rat brain. See *text* for further explanation. [1] Dross and Kewitz (13). [2] Dross and Kewitz (3), Eade *et al.* (14), Stavinoha and Weintraub (15). [3] Kewitz *et al.* (16). [4] Dross and Kewitz (3), Choi *et al.* (17). [5] Dross and Kewitz (13), Schuberth *et al.* (18), Pleul and Kewitz (19). [6] Goldman and Sapirstein (20).

level, since among all derivatives of Ch the specific activity of lecithin was highest provided the label injected had been attached to the methyl group of methionine or to EA and not to Ch.

In muscle we found no evidence of new synthesis of Ch.

It may be considered that free ethanolamine is the substrate for methylation in brain, whereas it is phosphatidylethanolamine in the liver.

Rates of transfer and transport have been calculated (Fig. 1) from measured pool sizes and experimentally established metabolic transfers of Ch and various of its derivatives in rat brain. For the calculation of transfer rates of Ch from blood to brain and vice versa the turnover rate of Ch in brain is needed. Since no measured values are available the order of magnitude of this rate can be established by trial and error, based on reasonable assumptions. A turnover rate of 32.5 nmol × g^{-1} × min^{-1} would lead to a transfer rate from blood to brain of 2.2 nmol × g^{-1} × min^{-1}. This would mean that the specific activity of Ch from blood would be diluted in brain by a factor of 15, which is almost three times as high as the dilution factor that we found, 5.6. A turnover rate as high as 42.5 nmol × g^{-1} × min^{-1} in turn would agree with a transfer rate from blood to brain of 12.2 nmol × g^{-1} × min^{-1}. Since only 10.8 nmol are transported with the arterial blood this figure must be too high. Thus, a reasonable turnover rate of Ch in brain may lie between these values, namely 36.5 nmol × g^{-1} × min^{-1}. Under this assumption 6.2 nmol × g^{-1} × min^{-1} are transferred from blood to brain, which is one-sixth part of the turnover rate, a figure that agrees well with the measured factor of 5.6 for the dilution of radioactive Ch from blood by unlabeled Ch in the brain. The demonstration of Ch synthesis in the brain appears to add a significant facet to the description of Ch metabolism in brain.

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