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Choline biosynthesis in sheep

Evidence for extrahepatic synthesis

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1. Choline production by various tissues of the sheep was measured by determining venous and arterial free choline concentrations in blood samples taken from various vessels in conscious multicannulated sheep. 2. Significant production of free choline occurred in the upper and lower body regions, and specifically in the heart, brain and hind-limb muscles of sheep, but there was no significant uptake or output of phosphatidylcholine across these tissues, as determined by arterio-venous differences. 3. In contrast, in the rat there were no significant arterio-venous differences in the concentrations of free choline or phosphatidylcholine across the hind-body. 4. Synthesis of phosphatidylcholine from endogenous phosphatidylethanolamine and *S*-adenosyl-L-[*methyl*-^{14}C]methionine was measured in experiments *in vitro* using microsomal preparations from a variety of sheep and rat tissues. 5. The biosynthetic activity was highest in liver from sheep and rats, although the activity in sheep microsomal preparations was about one-quarter of that in rat microsomal preparations. 6. Microsomal preparations from sheep lung, kidney, gut epithelium, brain, heart and skeletal muscles also showed considerable biosynthetic activity, but in the rat the activity was virtually confined to the liver. 7. Overall, the results show a significant production of choline in extrahepatic tissues of the sheep, with skeletal muscle contributing some 60% of this extrahepatic activity. Thus the extrahepatic production of choline in the sheep, together with the extensive reutilization of bile choline, can explain the maintenance of the large endogenous body pool of choline in this species.

INTRODUCTION

Sheep receive an insignificant amount of choline from the diet per day, as there is almost complete microbial destruction of dietary choline in the rumen (Neill *et al.*, 1978, 1979; Dawson *et al.*, 1981). Nearly all of the choline body pool in the sheep is of endogenous origin, whereas in rats 18–54% is of dietary origin (Dawson *et al.*, 1981). The capacity of sheep liver to synthesize choline appears to be less than that of rat liver (Bremer & Greenberg, 1961; Henderson, 1978; Neill *et al.*, 1979), and calculations have shown that hepatic synthesis only contributes about 18% of the endogenous choline body pool in sheep (Robinson *et al.*, 1984). Our previous studies, using conscious chronically cannulated sheep, showed that there is a major production of free choline by the upper and lower body regions drained by the superior and inferior vena cavae in the sheep (Robinson *et al.*, 1984). However, the specific organs or tissues producing this free choline were not identified.

The only known pathway for the synthesis *de novo* of choline in mammalian tissue involves the stepwise methylation of phosphatidylethanolamine to phosphatidylcholine with *S*-adenosylmethionine as the methyl donor, and is referred to as the methylation pathway. Although there is considerable debate as to whether one or more enzymes are involved in the three successive methylation reactions [e.g. see Hirata & Axelrod (1980) and Pajares *et al.* (1984) for opposing views], the entire pathway can be assayed *in vitro* under appropriate conditions (Bremer & Greenberg, 1961; Hirata *et al.*, 1978; Hoffman & Cornatzer, 1981; Sastry *et al.*, 1981).

The aim of the present investigation was to seek further evidence for the extrahepatic synthesis of choline in sheep and to identify the specific tissues involved. The experimental approach was at two levels: (*a*) at the whole-animal level, by using conscious multi-cannulated sheep, by measuring arterio-venous concentration differences in free and lipid choline across the upper and lower body regions, and specifically across the brain, heart and skeletal muscle; (*b*) *in vitro*, by using microsomal preparations from sheep and rat tissues to measure the synthesis of choline by the methylation pathway from endogenous phosphatidylethanolamine and ^{14}C-labelled *S*-adenosylmethionine.

EXPERIMENTAL

Animals

Merino sheep (ewes *Ovis aries*) of blood haemoglobin type A (determined by the method of Moore *et al.*, 1966), aged 1–2 years and weighing 30–45 kg, were used for conscious chronically catheterized preparations. They were given chopped lucerne hay and water *ad libitum*. Before surgery the animals were starved for 24 h.

Adult female Hooded Wistar rats (*Rattus norvegicus*) weighing 250–300 g were used. They were given water and a pelleted rat diet (Charlicks, Adelaide, South Australia, Australia) *ad libitum*.

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Surgical preparation and maintenance of conscious chronically catheterized sheep

Sheep were chronically catheterized with a combination of ascending aorta, superior vena cava, inferior vena cava, coronary sinus, sagittal sinus and hindlimb recurrent tarsal-vein catheters and maintained as described previously (Runciman, 1982; Runciman *et al.*, 1984; Robinson, 1985). Each sheep was allowed to stabilize on full feed intake for 7–10 days after surgery before blood samples were taken.

Collection of blood samples and determination of plasma unesterified choline and lipid choline

Blood samples (6 ml) were simultaneously withdrawn from respective catheters of conscious chronically prepared sheep at hourly intervals for at least 4 h. Blood samples (5 ml maximum) were drawn simultaneously from the inferior vena cava and descending aorta of rats (anaesthetized with diethyl ether) after abdominal incision. Blood was treated and plasma unesterified choline and lipid choline were determined in duplicate as described by Robinson *et al.* (1984) and Robinson (1985).

Collection of tissue samples and determination of microsomal phospholipid methylation

Sheep were slaughtered by severing their necks, and rats were killed by cervical dislocation and exsanguination. Tissue samples were rapidly excised and placed in ice-cold 0.25 M-sucrose/10 mM-Tris/HCl buffer (pH 7.4)/1.0 mM-$MgCl_2$. Various segments of the alimentary tract were initially irrigated with the ice-cold medium to remove digesta contamination. In some instances the alimentary-tract tissue was cut longitudinally and placed on an ice-cold glass plate, and the inner epithelium was collected into the medium by gentle scraping with a wooden spatula (Hübscher *et al.*, 1965).

Tissue homogenates were prepared and microsomal fractions isolated by differential centrifugation essentially by the method of Hoffman *et al.* (1981) and Sastry *et al.* (1981). Tissue (6 g) was minced with scissors in 30 ml of ice-cold 0.25 M-sucrose/10 mM-Tris/HCl buffer (pH 7.4)/1.0 mM-$MgCl_2$ and then homogenized for 1 min with a Polytron type PT 10 tissue homogenizer and sonication (Kinematica G.m.b.H, Luzern, Switzerland). The homogenate was filtered through four layers of gauze to remove cellular debris and then diluted with 18 ml of ice-cold homogenizing medium. The nuclear fraction was sedimented by centrifugation of the homogenate at 1000 *g* for 10 min. The postnuclear supernatant was centrifuged at 12000 *g* for 20 min to sediment the mitochondrial fraction. The microsomal and supernatant (cytosolic) fractions were prepared by centrifugation of the postmitochondrial supernatant at 95000 *g* for 70 min. All centrifugations were performed at 4 °C. The microsomal pellet was resuspended in 2–5 ml of ice-cold 0.25 M-sucrose/10 mM-Tris/HCl buffer (pH 7.4)/1.0 mM-$MgCl_2$ with a glass/Teflon hand-powered homogenizer (size A) (Arthur H. Thomas Co., Philadelphia, PA, U.S.A.) and stored at −15 °C until analysis. Phospholipid-methylation activity remained stable for several weeks under such storage conditions. However, upon repeated freezing and thawing about one-third of the activity was lost.

The protein concentration of tissue microsomal fractions was determined in duplicate by the micro-biuret method of Itzhaki & Gill (1964).

The overall methylation of endogenous phosphatidylethanolamine to phosphatidylcholine in tissue microsomal fractions was assayed by measuring the incorporation of the [^{14}C]methyl group from *S*-adenosyl-L-[*methyl*-^{14}C]-methionine into phospholipids. The procedure adopted was a modification of the methods described by Hoffman & Cornatzer (1981) and Sastry *et al.* (1981), using a high pH and a high concentration of radioactive *S*-adenosyl-L-methionine. Some of the general recommendations of Audubert & Vance (1983) were also followed. The incubation medium, in a 10 ml stoppered polyethylene centrifuge tube, contained 125 mM-Tris/HCl buffer (pH 9.3), 10 mM-$MgCl_2$, 0.1 mM-EDTA, 0.2 mM-*S*-adenosyl-L-[*methyl*-^{14}C]methionine (0.9 mCi/mmol) and microsomal fraction (1–4 mg of protein) in a total volume of 1.4 ml. The components of the incubation mixture were added as 1 ml of Tris/HCl buffer containing $MgCl_2$ and EDTA, 300 μl of microsomal fraction and 100 μl of *S*-adenosyl-L-[*methyl*-^{14}C]methionine. The reaction was initiated by the addition of radioactive *S*-adenosyl-L-methionine and the mixture incubated at 37 °C for 30 min. The reaction was stopped by adding 0.15 ml of HCl (11 M). The radioactive phospholipids were extracted by the addition of 4.25 ml of methanol/chloroform (5:2, v/v) containing the antioxidant 2,6-di-t-butyl-4-methylphenol (50 mg/l), followed by 1.5 ml of water and 1.5 ml of chloroform with intermediate mixing. The biphasic extract was shaken vigorously for 10 min and then centrifuged at 1000 *g* for 15 min. After the upper aqueous phase was aspirated and discarded, a 2 ml portion of the lower chloroform phase was transferred to a polyethylene scintillation vial and evaporated to dryness in a water bath at 80 °C for 1 h. The residue was redissolved in 3.5 ml of scintillation fluid [7 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)-benzene per litre of toluene/Triton X-100 (2:1, v/v)], and the radioactivity determined by counting for 10 min in a Packard Tri-Carb 460 CD liquid-scintillation system, which automatically corrected for quenching by the sample-channels-ratio technique. Heated (100 °C for 10 min) microsomal fractions were used as blanks. Total phospholipid methylation in microsomal fractions was measured in duplicate and expressed as pmol of [^{14}C]methyl groups incorporated into phospholipids/min per mg of protein. The conditions of the assay ensured near-optimum pH, a saturating concentration of *S*-adenosyl-L-[*methyl*-^{14}C]methionine, and linearity with respect to incubation time and microsomal protein. The [^{14}C]methylated phospholipids formed during the reaction were identified by quantitative t.l.c. of the assay chloroform phase (Prasad & Edwards, 1981). The [^{14}C]methylated phospholipid products accounted for approx. 90% of the total radioactivity in the assay chloroform phase, of which about 88%, 10% and 2% was associated with phosphatidylcholine, phosphatidyldimethylethanolamine and phosphatidylmonomethylethanolamine respectively. The radioactivity in phosphatidylcholine was specifically associated with the choline portion of the molecule.

Chemicals

These were obtained as follows: *S*-adenosyl-L-[*methyl*-^{14}C]methionine (sp. radioactivity 59 mCi/mmol and

Table 1. Unesterified choline concentrations in blood plasma from various blood vessels of conscious chronically catheterized sheep

At 1 h intervals blood samples were collected simultaneously from different vessels of conscious chronically catheterized Merino ewes. Unesterified choline was determined in plasma ultrafiltrates in duplicate as described in the text. The values shown are means ± S.E.M., with the numbers of blood samples taken from sheep given in parentheses.

	Unesterified choline concentration in blood plasma (nmol/ml)					
Sheep	Ascending aorta	Superior vena cava	Inferior vena cava	Coronary sinus	Sagittal sinus	Tarsal vein
1	4.2±0.5 (4)		5.3±0.5 (4)	5.6±0.4 (4)		
2	4.1±0.1 (8)				5.2±0.3 (8)	
3	2.5±0.1 (4)	3.8±0.3 (4)	3.4±0.2 (4)	2.7±0.2 (4)	3.6±0.2 (4)	
4	3.1±0.1 (4)		4.6±0.1 (4)			4.0±0.2 (4)
5	3.3±0.0 (4)		4.8±0.1 (4)			4.1±0.1 (4)
6	1.9±0.1 (5)				3.3±0.3 (5)	
7	4.4±0.2 (4)			5.2±0.1 (4)		5.5±0.2 (4)
8	4.8±0.3 (4)		6.4±0.5 (4)	5.6±0.6 (4)		
9	1.8±0.5 (4)		3.8±0.3 (4)	2.4±0.2 (4)	3.6±0.5 (4)	
10	2.5±0.4 (4)		3.0±0.3 (4)			
Mean	3.3	3.8	4.3	4.3	3.9	4.5

Table 2. Differences in blood plasma unesterified choline concentrations across various organs of conscious chronically catheterized sheep

The values shown are means ± S.E.M. for the numbers of blood samples taken from sheep, given in parentheses; + indicates net output by an organ. The significance of the differences across organs, as determined by Student's *t* test, is indicated. Abbreviations used: N.S., not significant; A, ascending aorta; SVC, superior vena cava; IVC, inferior vena cava; CS, coronary sinus; SS, sagittal sinus; TV, tarsal vein. (See Table 1 for absolute plasma unesterified choline concentrations in various vessels.)

	Differences in blood plasma unesterified choline concentration (nmol/ml)				
Sheep	Upper body region (SVC−A)	Lower body region (IVC−A)	Heart (CS−A)	Brain (SS−A)	Hindlimb muscle (TV−A)
1		+1.1±0.2 (+) $P < 0.02$	+1.4±0.2 (4) $P < 0.01$		
2				+1.1±0.3 (8) $P < 0.02$	
3	+1.3±0.3 (4) $P < 0.02$	+0.9±0.1 (4) $P < 0.01$	+0.2±0.0 (4) $P < 0,02$	+1.1±0.3 (4) $P < 0.05$	
4		+1.5±0.1 (4) $P < 0.001$			+0.8±0.1 (4) $P < 0.05$
5		+1.5±0.1 (4) $P < 0.001$			+0.8±0.1 (4) $P < 0.02$
6				+1.4±0.2 (5) $P < 0.01$	
7			+0.9±0.2 (4) $P < 0.02$		+1.2±0.3 (4) $P < 0.05$
8		+1.6±0.4 (4) $P < 0.05$	+0.8±0.8 (4) N.S.		
9		+1.0±0.7 (4) N.S.	+0.6±0.4 (4) N.S.	+1.8±0.3 (4) $P < 0.01$	
10		+0.6±0.2 (4) $P < 0.05$			
Mean	+1.3	+1.2	+0.8	+1.4	+1.0

radiochemical purity 98%) from Amersham Australia Pty. Ltd., Sydney, N.S.W., Australia; *S*-adenosyl-L-methionine (chloride salt, grade II) (used to dilute *S*-adenosyl-L-[*methyl*-^{14}C]methionine), 2,5-diphenyloxazole, 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene were from Sigma Chemical Co., St Louis, MO, U.S.A., and 2,6-di-t-butyl-4-methylphenol was from Calbiochem, Los Angeles, CA, U.S.A.

RESULTS

Unesterified choline concentrations in blood plasma from various blood vessels of conscious chronically catheterized sheep

Table 1 shows the blood plasma unesterified choline concentrations in six different blood vessels of ten conscious chronically catheterized sheep. Table 2 shows

Table 3. Lipid choline concentrations in blood plasma from various blood vessels of conscious chronically catheterized sheep

Blood samples were collected as described in the legend to Table 1. Plasma samples were extracted with chloroform/methanol, and lipid choline was fractionated and determined in duplicate as described in the text. The values shown are means ± S.E.M. for the numbers of blood samples taken from sheep, given in parentheses. There were no significant plasma concentration differences across organs (determined by Student's paired *t* test).

	Lipid choline concentration in blood plasma (nmol/ml)					
Sheep	Ascending aorta	Superior vena cava	Inferior vena cava	Coronary sinus	Sagittal sinus	Tarsal vein
1	404 ± 41 (4)		441 ± 30 (4)	439 ± 26 (4)		
2	325 ± 11 (8)				300 ± 11 (8)	
3	547 ± 12 (4)	556 ± 13 (4)	542 ± 8 (4)	534 ± 9 (4)	539 ± 4 (4)	
4	555 ± 14 (4)		574 ± 11 (4)			541 ± 13 (4)
5	548 ± 22 (4)		553 ± 25 (4)			550 ± 22 (4)
6	506 ± 22 (5)				496 ± 26 (5)	
7	411 ± 15 (4)			430 ± 16 (4)		422 ± 14 (4)
Mean	471	556	528	468	445	504

Table 4. Unesterified choline and lipid choline concentrations in blood plasma from the inferior vena cava and descending aorta of rats

Blood samples were collected simultaneously from the inferior vena cava and descending aorta of anaesthetized rats. Plasma samples were extracted with chloroform/methanol, and lipid choline was fractionated and determined in duplicate as described in the text. Unesterified choline was determined in plasma ultrafiltrates in duplicate. The values shown are means ± S.E.M. for the numbers of rats given in parentheses. The plasma concentration differences across the lower body region (IVC − A), as determined by Student's paired *t* test, were not significant.

	Choline concentration in blood plasma (nmol/ml)		
Form of choline	Inferior vena cava (IVC)	Descending aorta (A)	IVC − A
Unesterified	9.3 ± 0.9 (17)	9.5 ± 0.7 (17)	−0.2 ± 0.9 (17)
Lipid	848 ± 40 (10)	819 ± 38 (10)	+29 ± 25 (10)

the differences in blood plasma unesterified choline concentrations across various organs of the conscious chronically catheterized sheep. The results indicate significant output of plasma unesterified choline by the upper- and lower-body regions drained by the vena cavae and by the heart, brain and hindlimb muscle.

Lipid choline concentrations in blood plasma from various blood vessels of conscious chronically catheterized sheep

The blood plasma concentrations of lipid choline in different blood vessels of seven conscious chronically catheterized sheep are presented in Table 3. The values shown in the present paper are similar to those reported previously for sheep (Lindsay & Leat, 1975; Robinson *et al.*, 1984). No significant uptake or output of plasma lipid choline was detected across any organ of the seven animals.

Unesterified choline and lipid choline concentrations in blood plasma from the inferior vena cava and descending aorta of rats

The concentrations of unesterified choline and lipid choline in blood plasma from the inferior vena cava and descending aorta of rats are shown in Table 4. The values are consistent with those previously published for rat blood plasma (Ansell & Spanner, 1971; Wang & Haubrich, 1975; Zeisel & Wurtman, 1981; Jope, 1982; Hicks *et al.*, 1982). There were no significant differences in plasma unesterified choline and lipid choline concentrations from the inferior vena cava and descending aorta of the animals (Table 4). This supports the data of Dross & Kewitz (1972), which implied that there were no significant differences in whole-blood unesterified choline concentrations from the femoral veins and arteries of rats. The results suggest that there is no uptake or output of choline by the lower-body region of this species.

Methylation of phospholipids in microsomal fractions of sheep and rat tissues

The main product of the methylation of endogenous phosphatidylethanolamine under the assay conditions used was phosphatidylcholine. Although the liver was the major site of phosphatidylcholine synthesis in the sheep, the biosynthetic activity of microsomal fractions from sheep liver was only about one-quarter of that of rat microsomal fractions (Table 5). However, in the sheep appreciable synthesis also occurred in microsomal fractions from lung, kidney, gut epithelium, heart, brain and skeletal muscles (Table 5). In contrast, in the rat phosphatidylcholine synthesis was essentially confined to the liver.

DISCUSSION

Dawson *et al.* (1981) calculated that sheep require approx. 17.3 mmol of choline/day in order to maintain the body pool of choline, and indicated that this is almost entirely derived from endogenous origin. Robinson *et al.* (1984) have previously suggested that the liver contributes only about 18% of the total body pool of choline and that the other 82% appears to be supplied by tissues of the upper and lower body regions, which are drained

Table 5. Phospholipid methylation in the microsomal fraction of various sheep and rat tissues

Sheep and rats were slaughtered and tissue samples immediately collected. Tissue homogenates were prepared and microsomal fractions isolated by differential centrifugation. Phospholipid methylation and protein in microsomal fractions were determined in duplicate as described in the text. The values shown are means ± S.E.M. for three animals of each species. The significance of the difference between corresponding sheep and rat tissue microsomal fractions, determined by Student's *t* test, is indicated. Abbreviations used: N.S., not significant; N.D., not detectable; –, not determined.

	Phospholipid methylation (pmol of [^{14}C]methyl groups incorporated/min per mg of protein		
Tissue	Sheep	Rat	Significance (sheep versus rat)
Liver	17.2±1.0	64.0±2.7	$P < 0.001$
Lung	4.0±0.3	1.4±0.3	$P < 0.02$
Kidney	3.4±0.3	1.5±0.5	$P < 0.01$
Rumen	2.6±0.2*	–	
Abomasum or stomach	1.4±0.6*	1.0±0.2*	N.S.
Small intestine	2.7±0.3†	0.3±0.2*	$P < 0.01$
Large intestine	2.1±0.1*	0.8±0.3*	$P < 0.05$
Brain	1.1±0.6	0.8±0.3	N.S.
Heart	1.4±0.1	0.4±0.2	$P < 0.01$
Skeletal muscle (M. biceps femoris)	0.9±0.1	N.D.	

* Value is for the whole tissue wall and is similar for the inner epithelium.

† Value is for the whole duodenal wall and is similar for the whole jejunal and ileal walls. The inner epithelium of the regions of the small intestine also had an equivalent value.

by the venae cavae. Here we again confirm the production of choline by the upper and lower body regions, but more specifically show that heart, brain and skeletal muscle produce significant amounts of choline, as assessed by the measurement of venous minus arterial blood concentration differences across these organs. If these data are combined with blood flow rates across the organ, it is possible to calculate the production rates of unesterified choline by the organs, as shown in Table 6. Calculations for the combined upper and lower body regions show a production of 5.4 mmol of unesterified choline/day, of which 3.5 mmol (or approx. 60%) appears to arise from whole-body skeletal muscle, assuming that hindlimb muscle is representative of that of the rest of the body. The heart and brain only appear to contribute small amounts.

These whole-animal studies are supported by the experiments *in vitro* on the methylation of endogenous phosphatidylethanolamine by *S*-adenosyl-L-[*methyl*-^{14}C]methionine in microsomal preparations from various sheep tissues reported here. The experiments showed that sheep liver is considerably less active than rat liver in synthesizing phosphatidylcholine, confirming previous reports (Bremer & Greenberg, 1961; Henderson, 1978; Neill *et al.*, 1979). However, a number of extrahepatic tissues in the sheep, i.e. lung, kidney, brain, heart, skeletal muscle and epithelial tissue from a variety of parts of the alimentary tract, all showed significant activity for phosphatidylcholine synthesis. Although skeletal muscle showed a relatively low activity, it was still a significant amount; when the total body mass of skeletal muscle is taken into account, the biosynthetic capacity of the total skeletal muscle would be considerable. Recent experiments using intravenously administered [^{14}C]ethanolamine suggested that significant choline synthesis can occur in sheep intestinal tissue (R. M. C. Dawson, personal communication), confirming results presented here, although no synthesis in muscle tissues could be demonstrated by this approach. Aliev *et al.* (1980) reported that acetate is an important precursor for choline synthesis in various sheep tissues, although we could not repeat these findings (G. P. Xue, B. S. Robinson & A. M. Snoswell, unpublished work).

In contrast with this situation in sheep, there was no arterio-venous difference in free choline across the lower body region of rats, indicating a lack of production of free choline in that body region. In rats there is an efflux of free choline into blood across the brain (Dross & Kewitz, 1972; Choi *et al.*, 1975; Spanner *et al.*, 1976), but this makes only a very minor contribution to whole-body turnover. Further, in the experiments *in vitro* reported here, the liver was the only rat tissue to show appreciable methylation of phosphatidylethanolamine to phosphatidylcholine. Thus the present work confirms earlier work by Bremer & Greenberg (1961) and Bjørnstad & Bremer (1966), which showed that choline synthesis only occurs in the liver in the rat.

Finally, like choline synthesis, the synthesis of carnitine by the hydroxylation of γ-butyrobetaine occurs in skeletal muscle, heart, liver and kidney of sheep, but is confined to liver in rats (Erfle, 1975; Costa, 1977), thus emphasizing the differences in the site of biosynthesis of these important compounds in sheep and in rats.

The studies reported here, which showed considerable production of free choline in a variety of tissues of the sheep, raise the question as to the origin of the free choline. We have also shown here that, although there was a significant and consistent release of free choline across organs of the sheep, there were no significant differences in the concentrations of plasma phosphatidylcholine across these tissues, indicating that plasma free choline was not derived from the breakdown of plasma phosphatidylcholine. It appears from the work presented here that the most likely explanation is that the free choline released from tissues was derived from tissue phosphatidylcholine synthesized by the methylation pathway and subsequently hydrolysed by phospholipases or released by base exchange. The further finding reported here, that a variety of sheep tissues are capable of significant methylation of phosphatidylethanolamine, supports this view. Blusztajn *et al.* (1979) and Blusztajn & Wurtman (1981) reported that rat brain synaptosomal preparations can liberate free choline from phosphatidylcholine that is generated by methylation of phosphatidylethanolamine, which in turn is consistent with observations that there is a net efflux of free choline from rat brain (Dross & Kewitz, 1972; Choi *et al.*, 1975; Spanner *et al.*, 1976). Blusztajn & Wurtman (1981) suggested that specific physical domains of phospholipids may exist within synaptosomal membranes, such that

Table 6. Summary of output of plasma unesterified choline by organs of conscious chronically catheterized sheep

The mean output of plasma unesterified choline by an organ of the sheep was calculated by the product of the mean venous–arterial plasma unesterified choline concentration difference across the organ (from Table 2) and the organ blood flow rate.

Organ	Mean venous–arterial plasma unesterified choline (nmol/ml)	Blood flow rate (litre/min)	Mean production of plasma unesterified choline (mmol/day)
Upper body region	+1.3	1.50[a]	2.8
Lower body region	+1.2	1.50[a]	2.6
Heart	+0.8	0.17[b]	0.2
Brain	+1.4	0.13[c]	0.3
Hindlimb muscle	+1.0	0.22[d]	0.3
Whole body muscle	+1.0[f]	2.40[e]	3.5

[a]Total blood flow through venae-cavae-drained upper and lower body regions assumed to be equal to the cardiac output (5.41 litres/min) minus liver and kidney blood flows (1.46 and 0.95 litre/min) of sheep (Robinson, 1985). Thus blood flow through each region assumed to be half of total blood flow.

[b]Coronary blood flow calculated by using rate of 1.09 ml/min per g of sheep heart (Lindsay & Setchell, 1974) and sheep heart wt. of 0.4% of sheep body wt., i.e. 160 g for a 40 kg sheep (May, 1970).

[c]Brain blood flow calculated by using rate of 1.00 ml/min per g of sheep brain (Pappenheimer & Setchell, 1972) and sheep brain wt. of 130 g (May, 1970).

[d]Hindlimb-muscle blood flow calculated by using rate of 0.24 ml/min per g of sheep hindlimb muscle (Domanski *et al.*, 1974; Pethick *et al.*, 1981) and sheep hindlimb muscle wt. of 900 g (Domanski *et al.*, 1974).

[e]Whole-body muscle blood flow calculated by using rate of 0.24 ml/min per g of sheep hindlimb muscle (Domanski *et al.*, 1974; Pethick *et al.*, 1981) and sheep total muscle mass equal to 25% of sheep body wt., i.e. 10 kg for a 40 kg sheep (Pethick *et al.*, 1981).

[f]Assumed to be same as hindlimb muscle studied.

phosphatidylethanolamine methylation and phosphatidylcholine degradation occur in the same domain. There appear to be several phosphatidylcholine pools in rat brain synaptosomes: one with a half-life of 2 days, one with a half-life of 52.5 days (Pasquini *et al.*, 1973), and another which turns over much more rapidly and is formed by the methylation pathway (Blusztajn & Wurtman, 1981). Vance & Vance (1986) have presented evidence for a number of pools of phosphatidylcholine in rat hepatocytes. In regard to phosphatidylcholine degradation, Zeisel (1985) concluded that phospholipase A is the primary initiator of free choline release from phosphatidylcholine in rat brain membranes.

Thus it may well be that the release of free choline in sheep tissues occurs by a similar mechanism to that which occurs in rat synaptosomes, i.e. that phosphatidylcholine is synthesized from phosphatidylethanolamine by the methylation pathway and is then rapidly degraded by phospholipases to yield free choline. Obviously this aspect of choline turnover in sheep tissues requires further investigation.

In regard to the origin of the large endogenous body pool of choline in the sheep, we have demonstrated here that synthesis of phosphatidylcholine and release of free choline occur in a number of extrahepatic tissues of the sheep, and that from a quantitative aspect production by total skeletal muscle is quite considerable. However, taking this extrahepatic production into account, there is still a considerable discrepancy in the amount of choline required per day from endogenous sources, as calculated by Dawson *et al.* (1981). We have previously suggested (Robinson *et al.*, 1984) that in sheep choline lost in the bile as phosphatidylcholine appeared to be reabsorbed from the alimentary tract and returned to the liver via the portal blood. We have established (B. S. Robinson, A. M. Snoswell & B. P. Setchell, unpublished work) that this extrahepatic recirculation of choline from the bile is very significant in the sheep and that about 70% of choline in the bile as phosphatidylcholine is broken down to free choline and is reabsorbed and reutilized in bile synthesis in the liver. Thus the extrahepatic synthesis of choline in the sheep reported here, plus the enterohepatic reutilization of bile choline, can largely account for the large endogenous body pool of choline in sheep.

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