On the insensitivity of sheep to the almost complete microbial destruction of dietary choline before alimentary-tract absorption

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(Received 6 November 1980/Accepted 3 February 1981)

1. Injection of [*Me*-¹⁴C]choline into sheep indicated that the small amount of phosphatidylcholine present in abomasal digesta was largely (69%) of non-dietary or ruminal origin. 2. Long-term feeding of [*Me*-³H]choline to sheep produced insignificant labelling of plasma phosphatidylcholine, indicating that more than 99% of the choline body pool was of non-dietary origin. 3. In contrast, when rats were fed with [*Me*-³H]choline for similar periods, 18–54% of the tissue phosphatidylcholine was derived from dietary choline. 4. The loss of [¹⁴C]choline and ³²P from the plasma phosphatidylcholine after a single injection of these isotopes indicated a markedly slower turnover of choline in the sheep compared with the rat. This observation, coupled with a lack of liver glycerophosphocholine diesterase, provides an explanation for the insensitivity of the sheep to an almost complete microbial destruction of dietary choline before alimentary-tract absorption.

Choline present in the diet of a sheep is micro-biologically degraded within the rumen with its *N*-methyl groups being converted into trimethylamine and ultimately methane (Neill *et al.*, 1978). A small amount escapes destruction by being incorporated into the structural membranes of ciliate protozoa as phosphatidylcholine (Broad & Dawson, 1976). However, analysis of abomasal digesta emptying from the rumen has shown that, although the choline present is nearly all combined in lipid form, the amount is minimal and certainly would be, on a body-weight basis, inadequate for preventing pathological lesions in species sensitive to choline deficiency (Neill *et al.*, 1979).

In the present investigation it has been shown that even the minimal concentration of lipid choline present in the abomasal digesta is substantially of non-dietary origin. In addition on continuous feeding of sheep for long periods with radioactive choline, the lipid choline in the blood acquires virtually no labelling, which is in distinct contrast with similar experiments in the rat. We have also compared the turnover of plasma phosphatidylcholine in the sheep and the rat after the injection of single doses of [*Me*-³H]choline and [³²P]P_i. The results suggest that the turnover of the body choline pool in the sheep is about seven times slower than that in the rat, which provides a possible explanation for the insensitivity of the ruminant to choline deficiency.

Experimental

Collection and treatment of digesta samples from the sheep

Animals were fitted with cannulae in the rumen and abomasum and digesta samples were obtained as described previously (Neill *et al.*, 1979). Omasum contents were collected from slaughterhouse carcasses after tying off the rumen, reticulum and abomasum with ligatures. Where appropriate, lipids were extracted from the digesta (Neill *et al.*, 1979).

Collection of blood and extraction of lipids

Blood was collected from the jugular vein of the sheep into a heparin-containing syringe. It was collected from rats by cardiac puncture under diethyl ether anaesthesia. The blood was centrifuged and the clear plasma layer was removed. To 5 ml of plasma was added 180 ml of chloroform/methanol (2:1, v/v); the mixture was filtered through a glass-wool pad, and the filtrate was shaken with 0.2 vol. of water. The bottom layer was collected and taken to dryness.

Examination of lipids

Tissue lipids. Lipids prepared as described above or extracted from other tissues after homogenization [1 g and 20 ml of chloroform/methanol (2:1, v/v)] were dissolved in 10 ml of chloroform. A 0.8 ml portion of the plasma lipid or 0.5 ml of the tissue

extract was applied as a strip (2–3 cm) on a silica-gel t.l.c. plate (Merck F₂₅₄), which was developed in chloroform/methanol/water/acetic acid (60:60:5:1, by vol.). The phosphatidylcholine strip was located with I₂ vapour, scraped off and extracted with 5 ml of chloroform/methanol (1:1, v/v). A 1 ml portion was evaporated to dryness, taken up in scintillation fluid and its radioactivity was counted. A 2 ml portion was taken to dryness, the residue oxidized and phosphorus was determined (Neill *et al.*, 1979).

Digesta lipids. The highly-coloured lipid solution (obtained from 250 ml of digesta) was taken to dryness and dissolved in 10 ml of chloroform. The lipids were introduced on to an alumina column free of water-soluble alkali (20 cm × 2 cm diameter) poured in chloroform. The column was washed with chloroform until no further colour was eluted. The residual lipids including phosphatidylcholine were eluted with chloroform/methanol (1:1, v/v). The eluate was dried *in vacuo* and the residue was redissolved in 0.5 ml of chloroform. This was applied as a strip (2 cm) to a silica-gel plate (Merck F 254) prewashed chromatographically with diethyl ether. Pigments and non-polar lipids were swept to near the end of the run with a chloroform/methanol (9:1, v/v) solvent and, after drying, the phospholipids were separated with a second run in chloroform/methanol/water/acetic acid (60:60:5:1, by vol.). The phosphatidylcholine strip was located with I₂ vapour and extracted with 10 ml of chloroform/methanol (1:1, v/v); 8 ml was used for determining radioactivity and 1 ml for phosphorus determination as described above for blood lipids. In some long-term experiments the phosphatidylcholine was deacylated (Dawson *et al.*, 1962), but no evidence could be obtained of significant recycling of isotope into the fatty acid portion of the molecule.

Metabolism of choline by digesta samples

The samples of rumen fluid and abomasal contents were incubated anaerobically with [*Me*-¹⁴C]choline, and the production of radioactive volatile base was measured by the method of Neill *et al.* (1978). The semi-solid omasum contents were stirred beforehand with an equal volume of a bacteria-free supernatant obtained by centrifuging and Seitz-filtering rumen fluid. The latter filtrate was shown to be devoid of any ability to degrade choline.

*Feeding experiments with [*Me*-³H]choline*

Sheep. The daily doses of [*Me*-³H]choline were dispersed in ethanolic solution into vials and stored in a deep freeze. Inactive choline chloride (0.2 mg) was added as a carrier. Each day, small drops of the dose solution were spotted on to 1 kg of hay chaff, just before feeding at 10:00 h. The hay chaff was taken from a bulk well-mixed stock to maintain

consistency of choline administration. Rations were consumed within 3 h.

Rats. Albino rats (male; about 300 g) were individually caged and fed daily 14 g rat-cubes (Oxoid; stated choline content 137 mg/100 g). The appropriate daily dose of [³H]choline plus carrier in ethanolic solution had been spotted on to the cubes. The diet allowed (14 g) was entirely consumed by the rats during the 24 h period and provided much more choline than the nutritional requirement of the animal (Neill *et al.*, 1979).

Determination of the specific radioactivity of [³H]-choline in the foodstuff provided

Considerable purification was necessary before the specific radioactivity of the choline in the bulk food ration could be determined. A sample of hay or rat cubes (5 g) to which had been added [³H]choline and carrier choline in the same proportion as added to the animal's ration was ground in a mortar with 100 ml of 5% trichloroacetic acid solution and left for 1 h. The suspension was filtered through a pad of glass-wool and the solution was extracted three times with an equal volume of diethyl ether to remove the trichloroacetic acid. To the aqueous extract from hay was added an equal volume of ethanol and the copious precipitate was removed by centrifuging. The extract from rat cubes was taken to dryness, redissolved in 10 ml of water to which was added 50 ml of acetone; the precipitate was removed by centrifuging. The supernatants from either ethanol or acetone treatment were taken to dryness in a rotatory evaporator and the residue was dissolved in 5 ml of water, and adjusted to pH 7 with ammonia. The solution was introduced on to a column (20 cm × 2 cm diameter) of cation-exchange resin (Amberlite IR 120 H). The column was washed with water until free of acid and the choline was then eluted with 0.5 M-HCl (30 ml). The eluate was taken to dryness *in vacuo* and the residue was dissolved in 3 ml of water. Portions (0.1 and 0.2 ml) were spotted either alone or with choline standards on to paper chromatograms and developed in n-propanol/water/acetic acid (14:2:1, by vol.) (20 h descending). After thorough drying of the paper to remove all the acetic acid, the choline spots were located with I₂ vapour, marked and the I₂ allowed to vapourize from the paper. The spots were cut out, and the paper was eluted with 3 ml of water. A portion (1 ml) was mixed with 10 ml of scintillation fluid and its radioactivity was determined. A further portion (1.8 ml) was used for choline determination by the method of Appleton *et al.* (1953) as modified by Dawson (1956). It was repeatedly found that standard curves of choline recovered from paper chromatograms gave a linear response although the origin extrapolated not to zero but to 2.5 µg of choline. For the actual determination of choline,

therefore, paper chromatograms were set up that consisted of two standard choline curves added to either 0.1 or 0.2 ml of the Amberlite column eluate (see above). When plotted out these gave two standard curves of constant slope. The absorbance increment between the curves represented the choline in 0.2–0.1 ml of the eluate and the choline value was determined from the slope of the standard curves. Thus the specific radioactivity of [^3H]choline in the diet could be calculated.

Calculation of turnover time

In sheep, the specific radioactivities of [^{14}C]choline or [^{32}P] in plasma phosphatidylcholine can be fitted as a function of time to a single exponential curve ($Y = Y_0 e^{-kt}$, where Y and Y_0 are specific radioactivities at time t and t_0 respectively and k is a constant) for values of t between 7 and 28 days. The best estimate of half-life ($\ln 0.5/k$) was obtained by a direct-fit least-squares iterative technique (Deming, 1964) using a programmable calculator.

Since 'metabolic time' is proportional to (body wt.) $^{0.25}$ (Kleiber, 1975), the corresponding time for studies in the rat is roughly $(0.3)^{0.25}/40^{0.25}$ or 0.29 that is 2–8 days. In all studies, the regression accounted for 70–95% of the variance.

Radiochemicals

[$\text{Me-}^{14}\text{C}$]Choline (sp. radioactivity 40–60 Ci/mmol), [$\text{Me-}^3\text{H}$]choline (sp. radioactivity 5–15 Ci/mmol) and [^{32}P]P $_1$ (carrier-free) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Results

Origin of phosphatidylcholine in abomasal digesta of the sheep

Two sheep were injected intravenously with [$\text{Me-}^{14}\text{C}$]choline and serial samples of blood and abomasal digesta removed over the following 4 weeks. Analysis of the phosphatidylcholine isolated from the abomasal contents showed that this acquired significant radioactivity (Fig. 1), although with considerable day-to-day variation. Its specific radioactivity at times after 10 days amounted to some $68.6\% \pm 18.9$ (28 observations) of the same component in blood plasma. To determine whether this labelling of abomasal contents was due to direct secretion of phosphatidylcholine into the abomasum or recycling of radioactive choline entering the rumen via, e.g., saliva, equivalent observations were made in which the phosphatidylcholine of rumen contents was examined. The specific radioactivity of the phosphatidylcholine in the rumen contents was very low and in six observations made after allowing 20 days for isotopic equilibrium amounted to only 14.6% (4.2–27.7%) of that of the same phospholipid

Fig. 1. Labelling of phosphatidylcholine of blood plasma and abomasal digesta after choline injection into a sheep

The sheep was injected with 50 μCi of [$\text{Me-}^{14}\text{C}$]choline and measurements were made of the specific radioactivities of phosphatidylcholine isolated from the blood plasma (●) and abomasal digesta (○) at various time intervals.

in abomasal contents. Passage of phosphatidylcholine in rumen digesta to the abomasum could not account for the labelling of the phosphatidylcholine in abomasal digesta.

Contribution of dietary choline to body-pool choline in the sheep and rat

Although these experiments confirmed that dietary choline passing through to the abomasum was unlikely to make a significant contribution to the body choline pool (Neill *et al.*, 1979) the possibility existed that some body-pool choline was being derived by a rapid absorption of dietary choline directly through the rumen or omasum walls before microbial degradation. Consequently sheep were kept on a diet of hay, where the choline content is virtually all in the free form (Neill *et al.*, 1979) and to which had been added a daily allocation of [$\text{Me-}^3\text{H}$]choline (50–100 μCi). Blood samples were taken throughout the experiment and determinations were made of the specific radioactivity of the plasma phosphatidylcholine. Considering the large amount of radioactivity ingested by the sheep the label acquired by the plasma phosphatidylcholine was extremely low (3–12 d.p.m./μg of P; 15–45 days) and did not appear to be increasing significantly after 20 days. On comparison with the specific radioactivity of the choline fed, it could be calculated that no more than 0.44% of the choline in circulating phosphatidylcholine was derived from dietary choline. In a similar experiment performed with a different sheep a value of 0.41% was obtained.

Similar experiments were performed using rats, although to obtain sufficient tissue for analysis, individual rats were killed at each time point. Substantial labelling of the tissue phosphatidyl-

choline occurred, showing dietary choline was making a substantial contribution to the body pool (Table 1). After 23 days the contribution of dietary choline to soft-tissue labelling varied from 37 to 54% in plasma, liver, kidney, intestine and spleen, with somewhat less in the tissues known for their slower turnover of phospholipids, e.g. 28% in muscle and 18% in brain.

Destruction of choline by sheep ruminal, omasal and abomasal digesta

The previous experiments had suggested that in the sheep dietary choline makes an insignificant contribution to abomasal or body-pool phosphatidylcholine labelling. Yet it is known that choline-labelled protozoa re-introduced into the rumen do disappear, although at a somewhat slower rate than the general digesta (Coleman *et al.*, 1980). In addition microscopic examination of omasum con-

tents clearly reveals the presence of viable ciliated protozoa (G. S. Coleman, R. M. C. Dawson & D. W. Grime, unpublished work). This suggests that such protozoa undergo degradation in the omasum or abomasum with a breakdown of their phosphatidylcholine and subsequent destruction of the choline.

Consequently we have tested the ability of digesta removed from all three regions to bring about the breakdown of added choline with the production of trimethylamine. Such experiments showed clearly that both rumen fluid and omasum contents (diluted with rumen supernatant inactive at degrading choline) could substantially break down labelled choline, producing volatile base whereas abomasal contents were virtually devoid of such activity (Table 2). Analysis by g.l.c. of the volatile base in omasum contents showed that this contained trimethylamine.

Turnover of blood plasma phosphatidylcholine in the sheep and rat

In an attempt to ascertain the reason for the insensitivity of the sheep to the non-availability of dietary choline, measurements were made of the loss of labelled choline and [^{32}P]P_i from the plasma phosphatidylcholine after a single injection of these isotopes. There is reason to believe that for time

Table 1. *Tissue phosphatidylcholine labelling in rats fed daily with [Me- ^3H]choline*

Rats were fed 1.78 μCi of [Me- ^3H]choline and 14 g rat cubes daily. They were killed after various times and the phosphatidylcholine in their tissues was isolated and analysed. The specific radioactivity of the choline fed was calculated from direct determinations of choline mass and radioactivity of the diet and if the tissue phosphatidylcholine was all of dietary origin it would have a specific radioactivity of 412 d.p.m./ μg of P at equilibrium. Results for specific radioactivity are for individual animals.

Time (days)	Tissue	Specific radioactivity of phosphatidylcholine (d.p.m./ μg of P)	Contribution of dietary choline to tissue pool of phosphatidylcholine (%)
9	Plasma	135	33
		137	
		112	
16	Liver	137	30
		104	
		100	
23	Plasma	117	27
		109	
		234	
	Liver	210	54
		168	
		150	
	Intestine	159	37
		150	
		187	
	Kidney	203	47
		121	
		110	
	Muscle	121	28
		71	
		76	
	Brain	71	18
		76	
	Spleen	188	47

Table 2. *Volatile base produced from [Me- ^{14}C]choline on incubation with rumen, omasum and abomasum contents*

The digesta sample was incubated anaerobically with 1 μCi of [Me- ^{14}C]choline for 1.5 h at 38°C. The supernatant collected after centrifuging was analysed for total and volatile base radioactivity. Mean values $\pm$ S.E.M. are shown for the individual results for rumen fluid, and omasum and abomasum contents.

Sample	Radioactivity recovered as volatile base (% of original radioactivity)
Rumen fluid (30 ml)	83.3 80.0 12.4 15.2 72.2 86.6
Omasum contents (30 g) mixed with 30 ml of rumen fluid supernatant (free of bacteria)	58.4 47.0 65.1 81.5*
Abomasal contents (30 ml)	81.8* 2.1 0.6 1.1
Blank (30 ml of saline)	0.5
Rumen fluid supernatant (30 ml)	1.2

* Pasture-fed animals.

Table 3. *The turnover of the choline and phosphate moieties of phosphatidylcholine in the blood plasma of the sheep and the rat*

The turnover times were calculated as described in the text. Five separate sheep were used, and in three of these the turnover of phosphate and choline moieties were measured in the same animal after the simultaneous intravenous injection of [*Me*-¹⁴C]choline (125 μ Ci) and [³²P]P_i (5 mCi). Two separate experiments were made with rats. In each experiment a series of animals was injected intraperitoneally with 5 μ Ci of [*Me*-¹⁴C]choline and either 200 or 250 μ Ci of [³²P]P_i. For rats, average values are shown of the individual results and for sheep means $\pm$ S.E.M. are shown for the individual values.

Half-life of plasma phosphatidylcholine moieties (days)

	Choline	Phosphate
Sheep	23.8 13.2 16.5 9.5 14.6 } 15.5 $\pm$ 2.4	— 4.4 7.8 — 5.3 } 5.8 $\pm$ 1.04
Rats	2.05 2.07 } 2.06	1.67 2.78 } 2.22

periods measured in days the plasma phosphatidylcholine would be in, more or less, radioactive equilibrium with the liver pool and most of the other body tissues (Dawson, 1967). The liver is the source of the phospholipids in the plasma lipoproteins and is responsible for their turnover.

After an equilibration period of 4–5 days the choline and phosphate moieties of plasma phosphatidylcholine were found to turn over in a way that could be adequately described by a single exponential function, although the respective rate constants were very different. The mean half-life for choline of phosphatidylcholine was 15.5 days (five sheep) and for phosphate 5.8 days (three sheep) (Table 3). Two experiments were made using a series of rats injected with identical doses of [¹⁴C]choline and [³²P]P_i and killed at differing time intervals. The half-life for the phosphate moiety was 2.2, about 2.9 times less than the corresponding value for the phosphate moiety in sheep, which is the ratio expected from differences in 'metabolic time' due to differing body size. The choline moiety in rat plasma phosphatidylcholine had a turnover time about the same as the phosphate moiety. Thus the ratio of the turnover times of the choline moieties in rat and sheep was about 0.13, a difference much more than can be accounted for by differences in metabolic time.

Discussion

Neill *et al.* (1979) calculated that if all the choline present in the abomasal digesta (largely as phos-

phatidylcholine) was derived from dietary sources, the adult sheep could receive no more than 20–25 mg of the vitamin from the digesta passing into the abomasum. However, we also observed that when a sheep was defaunated to remove the phosphatidylcholine-containing ciliated protozoa from its rumen then the concentration of phosphatidylcholine in the abomasal digesta was above that in the ruminal contents. This suggested that some, at least, of the phosphatidylcholine present in the abomasal digesta was derived from non-dietary sources, e.g. from abomasal secretions or regurgitation of bile from the lower digestive tract.

The present experiments, which show that the phosphatidylcholine in abomasal contents becomes labelled after intravenous injection of [¹⁴C]choline, support this conclusion. This labelling cannot be due to recycling of radioactive choline by secretion into the rumen, e.g. saliva, since the ruminal phosphatidylcholine does not acquire significant labelling. Comparison of the labelling of phosphatidylcholine in the abomasal digesta and blood plasma indicates that, on average, only about one-third of the abomasal phosphatidylcholine can be derived from dietary sources. This means that through this route the sheep can derive less than 10 mg of the vitamin per day. This is some 100 times less on a body-weight basis than the minimum intake required to avoid pathological lesions in a species sensitive to low choline intake.

Presumably the very limited transfer of phosphatidylcholine present in the rumen protozoa to the abomasum is due to a selective retention of these micro-organisms in the rumen (Weller & Pilgrim, 1974; Bauchop & Clarke, 1976; Harrison, 1979; Coleman *et al.*, 1980). However, examination of the omasum contents taken from killed sheep clearly shows a considerable and viable population of rumen ciliated protozoa. It is possible that because of the unusual ionic conditions appertaining in this part of the digestive tract, protozoa die and their phosphatidylcholine is degraded by the powerful autolytic phospholipases present (Broad, 1974; Broad & Dawson, 1976). If this does occur it means that any free choline liberated must be rapidly lost from the digesta, since the concentration of free choline in abomasal contents is very low (Neill *et al.*, 1979). The omasal digesta seems to retain the very active microbial system for degrading choline that is present in the rumen; the abomasal digesta has entirely lost this capacity.

Since the possibility still existed that sheep could derive dietary choline through a rapid absorption before microbial destruction we have fed sheep on a hay diet in which the choline content (largely as free choline) had been labelled by the constant addition of [³H]choline. Virtually no labelling of plasma phosphatidylcholine (<0.5% of dietary choline

specific radioactivity) was noted after 46 days. Since in mammals the plasma phosphatidylcholine is derived from and removed by the liver (Dawson, 1967) it seems reasonable to believe that over long time intervals the extent of its labelling will provide a reasonable index of the labelling of the body choline pool that, on a mass basis, consists very largely of phosphatidylcholine. Indeed in our experiments with rats (Table 1), the specific radioactivity of plasma phosphatidylcholine is never below that of other tissues. Thus it can again be concluded from these experiments that in sheep dietary choline is an insignificant source for maintaining the body pool of choline.

This is in marked contrast with equivalent experiments with rats, where a sizeable proportion (18–54%) of phosphatidylcholine in the tissues examined became labelled with [^3H]choline after 23 days. This presumably is to be expected of any species known to be susceptible to a choline-deficient diet.

Using again the plasma phosphatidylcholine as an index of the labelling of the total choline body pool, it is apparent that a marked difference exists between the loss of choline labelling from this pool in the sheep and the rat. The results suggest that in the sheep the irretrievable loss of choline is seven times slower than in the rat on a whole-body-pool basis. Since choline is not known to be excreted to any significant extent in mammals, the loss is presumably through the choline oxidase system, which exists primarily in the liver. In fact, previous measurements on the short-term oxidation of [$\text{Me-}^{14}\text{C}$]choline injected into sheep suggested that its rate of oxidation to $^{14}\text{CO}_2$ was one-third of that in the rat (Neill *et al.*, 1979) and such results may have been distorted somewhat since the measurements were made (from economic necessity) before the injected choline had equilibrated with the total body pool. Presumably the substrate for choline oxidation in the liver must be partly derived by the catabolism of phosphatidylcholine via deacylation to glycerophosphocholine. The specific phosphodiesterase (EC 3.1.4.2) liberating choline from this latter intermediate is known to be almost non-existent in sheep liver and high in rat liver (Dawson, 1956). This factor could limit the oxidation of the liver choline pool in sheep and perhaps allow a supply of glycerophosphocholine to pass from the liver to other sheep tissues where the choline would then become available for replenishing the phosphatidylcholine pool in these tissues. Glycerophosphocholine is known to be in high concentration in sheep liver (Schmidt *et al.*, 1952) and presumably could be transported to other tissues via the blood.

Thus because of the decreased loss of choline from the system and the increased opportunities for recycling, the sheep has a minimal need for dietary

choline. In fact, in the normal animal almost the entire need for this vitamin must be satisfied by its biosynthesis through the methylation of phosphatidylethanolamine to phosphatidylcholine, which occurs predominantly in the liver (Bremer & Greenberg, 1961) and which is a reaction known to occur in the sheep (Neill *et al.*, 1979). Presumably tissue methionine is the ultimate source of the *S*-adenosylmethionine used in this methylation. At the measured rate of choline turnover, maintenance of the body pool of choline would require approx. 1.8 g of choline per day. Since we have estimated that 10 mg per day could be derived from dietary sources, this only amounts to 0.55%, a value in good agreement with the 0.44% and 0.41% derived from the long-term feeding experiments with [^3H]choline. The adult sheep would therefore need to use approx. 9 g daily of methionine for maintaining its body pool of choline. Since the calculated uptake of methionine in the small intestine of the sheep amounts to no more than 2 g/day (Egan & Macrae, 1979) this presumably means that the methyl group of the methionine used as donor for choline synthesis must be derived from one-carbon sources through tetrahydrofolate intermediaries.

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