DIETARY AND ENDOGENOUS LONG-CHAIN FATTY ACIDS IN THE INTESTINE OF SHEEP, WITH AN APPENDIX ON THEIR ESTIMATION IN FEEDS, BILE, AND FAECES

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Summary

Normal sheep and sheep with fistulae of the bile duct or the thoracic duct were used to provide quantitative information on the movement of fatty acids into and out of the intestine. The operation used to gain access to the thoracic duct did not cause any significant alteration in the absorption of either [^{14}C]tripalmitin injected into the rumen or [^{14}C]palmitic acid injected into the duodenum. Normal sheep absorbed the major fatty acids oleic ($92 \cdot 1 \pm 1 \cdot 3\%$), palmitic ($87 \cdot 3 \pm 5 \cdot 0\%$), and stearic acids ($93 \cdot 3 \pm 1 \cdot 4\%$) with almost equal efficiency, and the absorption of labelled tripalmitin injected into the rumen did not alter as the intake of fatty acids increased from 12 g/day ($90 \cdot 1 \pm 2 \cdot 3\%$) to 44 g/day ($90 \cdot 1 \pm 1 \cdot 3\%$).

An average of 6 g of fatty acids entered the duodenum each day in the bile, and an approximately equal amount entered from other endogenous sources. The rate of entry of fatty acids in the bile was increased by secretin and by infusion of bile into the intestine. Sheep deprived of bile absorbed $25 \pm 1 \cdot 6\%$ of labelled palmitic acid and slightly less of oleic and stearic acids but the transport of absorbed fatty acids in the lymph was negligible in the absence of bile.

I. INTRODUCTION

The plant materials ingested by grazing sheep may contain significant amounts of long-chain fatty acids, and these are predominantly unsaturated and frequently esterified (Hilditch and Williams 1964; Garton 1967). Up to 30 or 40 g of these dietary fatty acids may enter the rumen each day, but they are not absorbed in significant amount from the rumen (Heath 1963; Hilditch and Williams 1964; Garton 1967). Instead, the unsaturated fatty acids are hydrogenated and the esterified fatty acids liberated by hydrolysis before passing with the rest of the digesta to the intestine (Garton 1967).

Endogenous sources may also contribute significant amounts of fatty acids, and in rats may contribute 40% or more of the fatty acids in the thoracic duct lymph (Karmen, Whyte, and Goodman 1963; Baxter 1966; Shrivastava, Redgrave, and Simmonds 1967). In sheep, the bile contains a high concentration of phospholipids (Adams and Heath 1963; Lennox, Lough, and Garton 1965, 1968), but little quantitative information is available concerning the entry of fatty acids to the gut from the bile, or from microbial or epithelial cells, or from other endogenous sources.

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Similarly, little quantitative information is available concerning the absorption of fatty acids from the intestine in sheep. Observations have been made on the transport of fat in the intestinal lymph (Lascelles and Morris 1961; Heath and Morris 1962, 1963; Feliński *et al.* 1964), but these are of limited value in this regard because a significant proportion of the absorbed fatty acids do not enter the intestinal lymph in sheep (Heath and Morris 1962; Heath 1964). Some information is available from the studies of Heath and Morris (1962) on the absorption of labelled tripalmitin from the gut of sheep. However, these sheep had been operated on to produce lymphatic fistulae, and the effects of the surgical manipulations on the absorptive efficiency is not known. Similarly, it is not known whether the results obtained with tripalmitin accurately reflect the rate of absorption of the fatty acids, principally stearic, oleic, and palmitic, from the intestine (Carroll 1958; Carroll and Richards 1958; Lennox and Garton 1968).

The experiments described in this paper were designed to provide quantitative information on the absorption of fatty acids and on the contribution of endogenous fatty acids in sheep. Measurements have been made of the absorption of different labelled fatty acids, and of the effects of surgical manipulations on the absorption of fatty acids. Efforts have been made to assess both the role of bile in the absorption of fatty acids, and the contributions of endogenous fatty acids from biliary and non-biliary sources in sheep.

II. Materials and Methods

Merino or crossbred ewes or wethers that weighed between 25 and 43 kg were used for each experiment. They were kept inside in metabolism cages that were designed to enable collection of faeces uncontaminated by urine. Each sheep had continuous access to water, and was fed each day on 600 g of either lucerne or oaten chaff or, in one series of experiments, oaten chaff that contained 2, 4, or 6% maize oil.

Some of the sheep were operated on to remove the gall bladder, then a polyvinyl chloride cannula (Clear Vinyl Tube, Medical Grade, from Dural Plastics and Engineering Pty Ltd, Dural N.S.W.) was placed in the common bile duct to enable bile to be collected as it was produced (Heath and Morris 1963). Polyvinyl chloride cannulae were also placed in the abomasum or duodenum of sheep to enable lipids to be injected and to enable the return of bile to the gut. Lymph from the thoracic duct was collected by techniques that have been described by Lascelles and Morris (1961) and by Heath and Morris (1962). For each of the operations, which were all carried out under aseptic conditions, anaesthesia was induced with pentobarbitone sodium and was maintained with halothane in a closed circuit.

Lipids labelled with carbon-14 were obtained from the Radiochemical Centre, Amersham, England. Glyceryl tri-[1-^{14}C]palmitate was dissolved in commercial maize oil at the rate of 20 μCi/ml before being injected into the rumen, and it was washed into the rumen with 1 ml non-radioactive maize oil. [1-^{14}C]Palmitic acid, [1-^{14}C]oleic acid, and [1-^{14}C]stearic acid were each dissolved in oleic acid (May and Baker Ltd, Dagenham, England) at the rate of 10–50 μCi/ml, then injected through indwelling tubes into either the duodenum or abomasum. Each injection of labelled fatty acid was followed by an injection of 1 ml non-radioactive oleic acid through the indwelling tube. The exact amount of labelled oil injected in each experiment was determined by weighing the syringe before and after injection. Faeces were collected until they contained no radioactivity: this took 3–5 days after duodenal or abomasal injection and 5–7 days after ruminal injection.

Methods used for the extraction and estimation of fatty acids are given in the Appendix (see also Heath and Morris 1962). Each sample was extracted in triplicate, and for radioactivity analyses duplicate portions that contained 5–15 mg lipid were removed from each extract and

plated onto 3·1 cm^2 planchets. A thin end-window (1·5–2 mg/cm^2) Geiger–Müller tube was used to record 10^3 counts from each planchet, and appropriate corrections were applied for self-absorption.

The method of Irvin, Johnston, and Kopala (1944) was used to estimate the concentration of bile salts in the bile.

Student's *t*-tests and analyses of variance were used to estimate the significance of differences between means in the different experimental groups.

III. Results

(*a*) *Absorption of Major Fatty Acids*

The absorption of oleic, stearic, and palmitic acids was studied in two sheep that had been operated on several weeks previously to place indwelling tubes in the abomasum. A measured amount (about 20 μCi) of ^{14}C-labelled fatty acid dissolved in non-radioactive oleic acid was injected into the abomasum and the fatty acids that were not absorbed were extracted from the faeces and the level of radioactivity measured. Estimated percentage absorption values (in duplicate) for both sheep were as follows:

	Sheep 1		Sheep 2	
Oleic acid	95	94	91	90
Palmitic acid	96	93	94	90
Stearic acid	81	96	77	99

About 90% of each of the fatty acids was absorbed, and no significant differences existed between the values obtained for the different acids (variance ratio 1·10; degrees of freedom 2, 8) or between the values obtained for the different sheep (variance ratio 0·84; degrees of freedom 1, 8).

(*b*) *Effect of Variations in Dietary Fat Intake*

The absorption of dietary fat and of ^{14}C-labelled fat injected into the rumen was studied in four trials in sheep that received different dietary intakes of fatty acids. In each trial, four sheep that weighed 25–30 kg were fed on 600 g of either oaten chaff or oaten chaff mixed with 2, 4, or 6% maize oil. The oaten chaff contained 20 mg fatty acids/g and the maize oil 910 mg fatty acids/g: the diets contributed between 12 and 44 g fatty acids each day (Table 1). In preliminary trials, it was found that if more than 6% maize oil was added, the chaff became unpalatable and was rejected by the sheep.

When each sheep had been eating a particular diet for 5 days, it received an injection of 10 μCi [^{14}C]tripalmitin in 0·5 ml maize oil into its rumen through a long 14 s.w.g. needle. The labelled fatty acids that were excreted in the faeces were recovered and estimated, and, in addition, the total output of long-chain fatty acids in the faeces was measured each day for 5 days (see Appendix).

These sheep absorbed slightly more than 90% of the labelled fat, and this percentage remained constant despite variations in the dietary fat intake (Table 1). As the dietary fat intake increased, increases did occur in the faeces in the concentration and output of fatty acids ($P < 0·05$). The apparent digestibility of the dietary fat also increased, and this approached the percentage absorption of the labelled fat (Table 1).

When the rate of excretion of fatty acids derived from the diet was estimated from the dietary fat intake and the percentage excretion of labelled fat, it was found to be significantly related to the dietary fatty acid intake ($P < 0{\cdot}01$):

$$D = 0{\cdot}04 + 0{\cdot}076I,$$

$$T = 1{\cdot}56 + 0{\cdot}078I,$$

where D is the faecal output of dietary fatty acids (g/day), T is the faecal output of total fatty acids (g/day), and I is the dietary fatty acid intake (g/day). At any level of dietary intake, the total fatty acid output in the faeces was about 1·5 g/day higher than the faecal output of fatty acids derived from the diet, and this was believed to represent the endogenous fatty acids that had not been absorbed. Variations in the intake of fatty acids in the diet did not appear to be associated with any progressive alterations in the rate of excretion of endogenous fatty acids.

TABLE 1

ABSORPTION OF FAT BY SHEEP FED DIETS CONTAINING DIFFERENT AMOUNTS OF FAT

Each sheep consumed 600 g of diet each day

Diet	Absorption of [^{14}C]Tripalmitin* (%)	Dietary Fatty Acids		Faecal Fatty Acids		Apparent Digestibility of Dietary Fatty Acids (%)†
		Concn. (mg/g)	Intake (g/day)	Concn. (mg/g dry wt.)	Output (g/day)	
Oaten chaff	90·1±2·3	20	12	12·0±1·0	2·7±0·5	78·5±4·2
+2% maize oil	90·6±1·7	38	23	14·7±1·4	3·6±0·2	84·6±0·9
+4% maize oil	91·9±0·9	56	34	15·7±2·1	4·2±0·8	87·5±2·2
+6% maize oil	90·1±1·3	74	44	23·9±6·4	5·8±2·1	86·9±4·6

* A solution (0·5 ml) of [^{14}C]tripalmitin (10 μCi) in maize oil was injected directly into the rumen and the radioactivity in the faecal excretion measured.

† Apparent digestibility $= \dfrac{100\,(\text{fatty acid intake} - \text{fatty acid output})}{\text{fatty acid intake}}$.

Experiments were then carried out on these endogenous fatty acids, particularly those derived from the bile, and an effort made to determine some of the factors that affect their rate of entry into the gut.

(c) *Entry of Endogenous Fatty Acids in Bile*

The contribution by the bile to the fatty acids in the intestine was estimated in four sheep that had tubes placed in the common bile duct and duodenum. The bile was collected for periods of 24 hr and, after measuring and sampling, was pumped back into the duodenum during the next 24 hr. The mean bile flow during a period of 4 days varied between sheep from 367 to 854 ml/day (Table 2). This bile contained about 1 g fatty acids/100 ml and contributed means of 3·0–7·7 g fatty acids to the gut each day in the different sheep: the mean daily output for the four sheep was 6 g. When the return of bile to the gut was discontinued, the bile flow decreased and a significant decrease occurred in the output of fatty acids in the bile (Table 2).

The effect of secretin and of bile on the output of biliary fatty acids was measured in three sheep that had tubes in the duodenum and common bile duct and from which the gall bladder had been removed, so that short-term changes in bile flow and composition could be studied. The bile was not returned to the gut of these sheep continuously, but a solution of sodium chloride (120 mM), sodium bicarbonate (18 mM), and potassium chloride (7 mM) was infused into the duodenum at about the rate of bile flow. When this solution but not bile was infused into the duodenum, the bile flowed at an average rate of 0·40 ml/min and contained an average of 4·5 mg fatty acids/ml; 1·8 mg fatty acids entered the duodenum each minute.

TABLE 2

CONTRIBUTION OF ENDOGENOUS FATTY ACIDS BY BILE

Bile was collected for four periods each of 24 hr and, after measuring and sampling, was pumped back into the duodenum during the next 24-hr period

Sheep No.	Return of Bile to Gut	Bile Flow (ml/day)	Bile Fatty Acid Concn. (mg/ml)	Bile Fatty Acid Output (g/day)
1	Returned	854±22	8·3±0·23	7·1±0·21
	Discontinued	330±2·2	11·7±0·48	3·9±0·15*
2	Returned	428±82	11·0±1·3	4·4±0·50
	Discontinued	238±15	12·0±0·34	3·0±0·21*
3	Returned	367±30	12·8±0·94	4·7±0·58
4	Returned	772±57	10·4±0·70	7·7±0·34

* The fatty acid output in the bile was significantly lower during bile deprivation than during the periods of bile return (sheep 1, $P < 0·001$; sheep 2, $P < 0·05$).

The rate of entry of fatty acids in the bile increased when 50 units of secretin (Boots Pure Drug Co. Ltd, Nottingham, England) was injected through an indwelling cannula into the jugular vein (Fig. 1). This was associated with an increase in the bile flow, but no consistent changes occurred in the concentration of fatty acids in the bile. The increase that occurred in the output of fatty acids began during the first 10 min and appeared complete within 30 min after the injection of secretin. A similar but much more prolonged increase in biliary fatty acid output occurred after the infusion of 500 ml bile into the duodenum over a period of 30 min. The bile flow and the concentration and output of bile salts also increased substantially after the bile was given (Fig. 1), but the changes that occurred in the fatty acid concentration in the bile were variable.

(*d*) *Absorption of Fatty Acids during Bile Deprivation*

The dietary intake and faecal output of fatty acids and the percentage absorption of ^{14}C-labelled fatty acids were studied in experiments on sheep deprived of bile by means of biliary fistulae.

In the absorption experiments, labelled acid was injected through an indwelling tube to the duodenum or abomasum (see Section II). Two sheep absorbed an average of 24·5% of the [^{14}C]palmitic acid (individual values 22·4 and 26·6%) injected into the duodenum, and another two sheep an average of 25·5% (individual values 28·7 and 22·3%) of that injected into the abomasum. In other experiments, 13·4 and 9·0% [^{14}C]oleic acid and 6·1 and 23·1% [^{14}C]stearic acid were absorbed from the intestine. As oleic, stearic, and palmitic acids comprise the bulk of the fatty acids in the intestine (Garton 1967), these results indicate that approximately one-sixth of the intestinal fatty acids are absorbed in sheep deprived of bile.

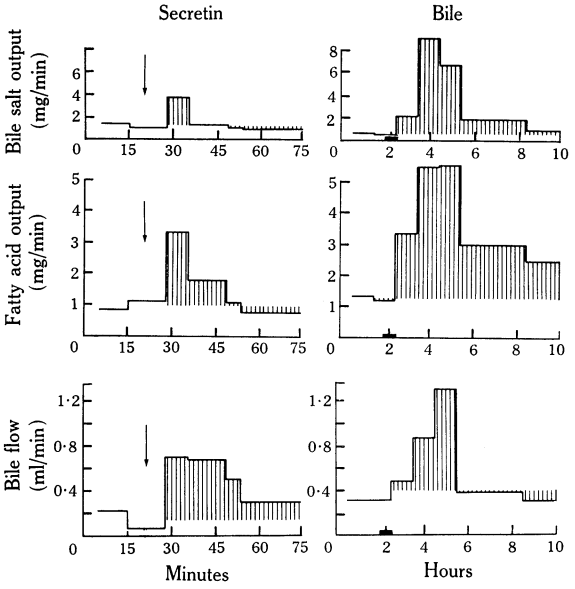

Fig. 1.—Bile flow and output of fatty acids and bile salts in typical sheep that received either 50 units secretin (arrow) through a jugular cannula, or 500 ml bile (black area) through an indwelling duodenal tube. Shaded areas represent changes from control values. The gall bladder was removed from each sheep, which was also deprived of bile, but a solution of 120 mM sodium chloride, 18 mM sodium bicarbonate, and 7 mM potassium chloride was infused into the duodenum at about the rate of bile formation.

Although each of the sheep ingested 600 g lucerne chaff per day in the dietary intake and faecal output experiments, wide variations existed in fatty acid concentrations between samples, and the intake of fatty acids by sheep used for the studies on palmitic acid absorption varied between approximately 12 and 24 g/day, as shown in the following tabulation:

Sheep No.	Dietary Intake of Fatty Acids (g/day)	Faecal Output of Fatty Acids (g/day)	
		Total	Dietary
1	11·6	13·9	10
2	11·6	13·4	10
3	18·9	18·9	16
4	24·3	21·1	20

If it is assumed that these sheep absorbed one-sixth of the dietary fatty acids, it can be calculated that the rate of excretion of dietary fatty acids was an average of 3 g/day less than the total output of fatty acids in the faeces (see above tabulation). This is believed to represent the endogenous fatty acids that were not absorbed.

In another series of experiments, a thoracic duct fistula was established in a sheep that was deprived of bile, and the total concentration of long-chain fatty acids in lymph and plasma was compared. In each case, the lymph concentration ($4 \cdot 6 \pm 0 \cdot 47$ μ-equiv/ml) was less than that of the plasma ($7 \cdot 9 \pm 1 \cdot 5$ μ-equiv/ml). When [^{14}C]palmitic acid was injected into the abomasum of this sheep, 32% was absorbed, but only $1 \cdot 7$% of the labelled fatty acid appeared in the lymph.

(e) *Effect of Surgery on Absorption of Fatty Acids*

Many experiments in this study and in previous studies were on sheep that were exposed to surgical manipulations (Heath and Morris 1962, 1963; Heath 1963; Heath, Adams, and Morris 1964). For this reason, experiments were designed to determine the effects, if any, of these operations on the absorption of fat. Five sheep that were fed on 600 g/day of lucerne chaff received an injection of [^{14}C]tripalmitin directly into the rumen, and the total amount of labelled fat excreted in the faeces was measured. Each sheep was then anaesthetized with pentobarbitone and maintained with halothane in closed circuit while a thoracotomy was performed. The surgical technique used involved removal of the eighth rib on the right side, and was the same as that used during cannulation of the thoracic duct by the approach of Lascelles and Morris (1961). Each operation lasted 30–45 min, and shortly after its completion a second injection of labelled tripalmitin was given into the rumen. When the radioactivity of the faeces was estimated it was found that the operation did not cause any significant alteration in the percentage absorption of the labelled fat [before surgery $89 \cdot 5 \pm 1 \cdot 3$% absorption, after surgery $91 \cdot 8 \pm 1 \cdot 0$%, $t = 1 \cdot 4$ (not significant) for 4 degrees of freedom].

In a second series, [^{14}C]palmitic acid was injected into the duodenum of five sheep which ingested 30 g fatty acids each day and the percentage of the injected radioactivity was measured in the faeces. In addition, the dietary intake and faecal output of fatty acids was measured each day for 3 days. After this time, each sheep was subjected to a thoracotomy and then, within 6 hr after the end of the operation, to a second injection of labelled palmitic acid. It was found that the percentage of labelled fatty acid that was absorbed was the same as that absorbed before the operation. These sheep each ingested 600 g lucerne chaff that contained 30 g fatty acids each day, and no significant alterations in either the concentration or the output of fatty acids in the faeces occurred as a result of the operation. These results are summarized in the following tabulation:

	Absorption (%)	Concn. in Faeces (mg/g dry wt.)	Output in Faeces (g/day)
Before surgery	$77 \cdot 9 \pm 7 \cdot 0$	$36 \pm 1 \cdot 7$	$6 \cdot 7 \pm 0 \cdot 5$
After surgery	$77 \cdot 5 \pm 8 \cdot 1$	$44 \pm 3 \cdot 6$	$8 \cdot 4 \pm 1 \cdot 0$
t (4 degrees of freedom)	0·04 (n.s.)	2·1 (n.s.)	1·5 (n.s.)

IV. Discussion

The sheep used in these experiments absorbed about 90% of each of the fatty acids studied, and the percentage absorbed was not altered by variations in the dietary fat intake, or by the imposition of a mild surgical stress. The fatty acids that

were studied, palmitic, oleic, and stearic, are the principle fatty acids in the lumen of the intestine, and they are derived either from the diet after hydrogenation in the rumen or from endogenous sources (Garton 1967; Lennox, Lough, and Garton 1968). Each of these fatty acids is present in significant amount in the lymph from the intestine in sheep (Feliński *et al.* 1964; Heath, Adams, and Morris 1964), but the results of experiments on sheep with re-entrant intestinal cannulae indicate that oleic acid may be absorbed a little more efficiently than the saturated fatty acids (Lennox and Garton 1968). It was not possible to detect any significant differences between the absorption of the different ^{14}C-labelled fatty acids used in the current study.

In non-ruminant animals, however, marked differences have been reported between the absorption of oleic acid on the one hand and stearic and palmitic acids on the other hand, and in the experiments of Carroll (1958) and Carroll and Richards (1958) less than one-quarter of the stearic acid in the intestine of rats was absorbed. It is likely that the physical properties of the saturated acids may be partly responsible for these low values, and that in sheep the relatively slow and continuous release of digesta into the duodenum may facilitate their incorporation into micelles and subsequent absorption (cf. Feliński *et al.* 1964). It is also possible that variations exist between species in the structure and composition of micelles in the intestine, and that these variations may be related to the relatively high intestinal pH, the low concentration of monoglycerides, and the high concentration of phospholipids in the intestine in sheep (Hofmann 1966; Bath and Hill 1967; Garton 1967; Lennox, Lough, and Garton 1968).

The deprivation of bile would be expected to decrease the formation of these micelles, and to decrease the absorption of fatty acids. In rats deprived of bile, the absorption of fatty acids is depressed to between one-half and three-quarters of the normal value, but a number of authors have been able to recover only a small percentage of these absorbed fatty acids in the lymph (Borgström 1953; Saunders and Dawson 1963; Gallagher, Webb, and Dawson 1965). Dawson's group demonstrated that some absorbed oleic acid was transported in the portal blood in rats deprived of bile (Saunders and Dawson 1963), and later suggested that long-chain fatty acids may be predominantly transported in the portal vein in animals deprived of bile, and to some extent in normal animals (Gallagher, Webb, and Dawson 1965). Morgan (1966) questioned this point of view and suggested that under certain conditions bile fistula rats can transport most if not all of the absorbed fat in the lymph. Morgan found a very much higher percentage of absorbed fatty acids in the lymph of bile-deprived rats tested 48 hr after the operation than in those tested during the first day, and suggested that the small lymphatic recovery of absorbed fatty acids by other workers may have been related to some metabolic effects of the operative trauma.

When sheep that are clinically normal and apparently have recovered from the immediate effects of the operation are deprived of bile, about one-sixth of the fatty acids in the intestine are absorbed [see Section III(*d*)]: the depression in fat absorption is much greater than in monogastric animals (Bernhard, Ritzel, and Hug 1952; Saunders and Dawson 1963). Within 10 hr after the diversion of bile from the intestine of these sheep the lymph contains a lower concentration of fatty acids than the plasma (Heath and Morris 1963). It is believed that almost all of the

fatty acids present in the intestinal lymph in the absence of bile are derived from the plasma, and that the contribution from the intestinal lumen is negligible. This was confirmed in the current study when labelled palmitic acid was injected into the gut of a sheep deprived of bile and less than 2% was recovered in the lymph. However, in this and other experiments significant amounts of labelled fatty acids were absorbed in sheep deprived of bile. In sheep that are not deprived of bile a significant proportion of the absorbed fatty acids is not recovered in the lymph (Heath and Morris 1962; Heath 1964) and is probably transported in the portal vein. The diversion of bile would appear to be followed by an increase in the relative importance of the portal vein in the transport of those fatty acids that are absorbed.

Bile is also important as a source of endogenous fatty acids, and contributed an average of 6 g/day to the sheep. It is likely that the biliary output of fatty acids varies depending on the level of secretin and on the resorption of biliary constituents (Fig. 1; Table 2), and that most of the fatty acids are present in phosphatidyl choline (Adams and Heath 1963; Lennox, Lough, and Garton 1968). Endogenous fatty acids may also be derived from microorganisms and desquamated cells, and possibly by direct secretion across the wall of the intestine (Leblond and Stevens 1948; Habel 1959; Burr, McPherson, and Tidwell 1960; Ward, Scott, and Dawson 1964). These sources contributed an average of 3 g/day of faecal fatty acids in sheep with biliary fistulae [Section III(*d*)]. This value is rather lower than that expected from the experiments on intact sheep of similar size. These sheep excreted about 1·5 g/day of endogenous fatty acids derived from both biliary and non-biliary sources (Table 1). The experiments on absorption of different fatty acids indicate that about 90% of the fatty acids entering the intestine would be absorbed (see tabulation, p. 1017; also Table 1), and that 1·5 g faecal fatty acids would be derived from about 15 g endogenous fatty acids. The difference between these estimates may be due in part to the fatty acids produced by the bacteria of the large intestine (Ward, Scott, and Dawson 1964), as these would not have been presented for absorption in the small intestine.

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APPENDIX

ESTIMATION OF LONG-CHAIN FATTY ACIDS IN FEEDS, BILE, AND FAECES

By T. J. HEATH

To date, no method has been available which is suitable for the direct measurement of the fatty acid content of feeds and faeces of experimental animals. In most previous experiments with animals, the lipid content of feed and faeces has been estimated gravimetrically after extraction of the sample with ether (Lucas and Loosli 1944; Association of Official Agricultural Chemists 1965), but ether extracts a large amount of pigment in addition to fat. Furthermore, results of preliminary experiments in the present study showed that the concentration of ether-extractable material may bear little relation to the concentration of fatty acids and fatty acid esters in the sample extracted (Table 3).

TABLE 3

COMPARISON BETWEEN LEVELS OF FATTY ACIDS AND ETHER-EXTRACTABLE MATERIAL IN FEED AND FAECES OF SHEEP FED DIFFERENT AMOUNTS OF FAT

Diet	Method of Estimation	Dietary Fatty Acid Concn. (mg/g)	Total Fatty Acid Input (g/day)	Faecal Concn. (mg/g dry wt.)*	Faecal Output (g/day)*
Oaten chaff	Ether extract†	54	32	57	11
	Fatty acids	20	12	12	2·7
Oaten chaff +6% maize oil	Ether extract†	107	64	56	11
	Fatty acids	74	44	24	5·8

* Values are means from experiments on four sheep which consumed either 600 g of oaten chaff or 600 g oaten chaff with 6% maize oil each day.

† 0·5–1 g portions were refluxed for 4 hr with 30 vol. of ether.

A description is here given of a precise analytical method for the estimation and fractionation of long-chain fatty acids in feed and faeces and also in bile. Methods

are given for the extraction of the lipids from feed and faeces before and after drying, and for their separation on thin-layer chromatograms. The lipids in the original sample or in chloroform–methanol extracts or in eluates from chromatograms are hydrolysed, and converted into copper soaps. The concentration of these soaps is estimated colorimetrically after reaction with diethyldithiocarbamate (Duncombe 1963, 1964).

Materials

Samples of feed were obtained from feed merchants. Faeces were obtained from sheep that were fed either lucerne chaff, oaten chaff, or oaten chaff that contained 6% maize oil. Some of these sheep had been operated on to produce bile fistulae (Heath and Morris 1963), but these fistulae were arranged so that the bile could be returned to the duodenum at will. Faecal samples were collected during bile deprivation, and bile samples from animals not deprived of bile. Faeces were also obtained from rabbits in which the bile duct had been ligated and [^{14}C]palmitic acid injected into the stomach.

Reagent-grade chemicals (May & Baker Ltd.) that were not repurified were used for almost all estimations, but some lipid standards were obtained from the Sigma Chemical Co., St. Louis. [^{14}C]Palmitic acid, used in testing steps in the method, was obtained from the Radiochemical Centre, Amersham.

Solvent Extraction of Feed and Faeces

Finely ground samples of feed and faeces were dried overnight at 110°C, then *c.* 1-g portions were refluxed with about 30 ml of chloroform–methanol–conc. HCl 3 : 2 : 0·05 v/v) for 1 hr. Tests to determine the effects of time of extraction, drying the sample, and varying the volume and methanol content of the solvent were conducted on faeces containing radioactive fatty acids. These were obtained from rabbits with ligated bile ducts which had received intragastric injections of [^{14}C]palmitic acid.

Experiments in which dried samples of faeces were refluxed in chloroform–methanol (3 : 2 v/v) for different periods showed that levels of fatty acids in portions extracted for 30 or 60 min were not less than those in portions extracted for 9 hr and allowed to stand overnight.

Although it was possible to extract lipids with chloroform–methanol from samples which had not been dried, the efficiency of extraction from the non-dried faeces after refluxing for 1 hr was significantly less than from the dried samples ($P < 0{\cdot}01$), and a significant interaction existed between the state of the sample (wet or dry) and the time of extraction ($P < 0{\cdot}05$). Wet samples required a longer time of extraction, hence the period of refluxing was increased to 4 hr in these cases. Neither the volume (25 or 12·5 ml) nor the composition of the solvent (3 : 2 or 2 : 1 chloroform–methanol) had any significant effect in this trial.

Fractionation of Lipids by Thin-layer Chromatography

In some cases, the lipid fractions in an extract were separated by thin-layer chromatography on prepared sheets of silica gel (Eastman Chromogram Sheets 6060; Distillation Product Industries, Rochester, N.Y.). Two ml of chloroform–methanol extract were taken to dryness in a stream of nitrogen, then the lipids were dissolved in 0·2 or 0·4 ml chloroform. A measured volume (100–200 μl) of the concentrated

extract and of standard solutions was applied in a line to 20-cm sheets of silica gel that had been activated at 105°C for 30 min.

The plates were developed in ether–benzene–ethanol–glacial acetic acid (40 : 50 : 2 : 0·2 v/v) (Freeman and West 1966) and then dried. This solvent system allowed separation of partial glycerides, but cholesterol esters were not separated from triglycerides. These could be separated on longer plates by a second run in diethyl ether–hexane (6 : 94 v/v) as described by Freeman and West (1966). The phospholipids remained at the origin, but these could be separated if chloroform–methanol–water (80 : 25 : 3 v/v) was used (Vogel, Doizaki, and Zieve 1962).

The positions of the fractions were determined with iodine vapour which was then allowed to sublime. The areas of silica gel that contained each lipid fraction, and an area with no lipid that could provide blank values, were scraped into separate 5-μl syringes in which the needle holes had been covered with lipid-extracted filter paper, and one drop of glacial acetic acid was added. Free fatty acids were eluted with 8 ml chloroform, but fatty acid esters were eluted with 5 ml ethanol and hydrolysed as described in the following section before the fatty acids were extracted into chloroform.

Hydrolysis of Fatty Acid Esters

(i) *Feed and Faeces*

About 4–8 ml of chloroform–methanol extract was used, the solvent being first removed with a stream of nitrogen and 5 ml ethanol added. Fatty acid esters eluted with ethanol after fractionation on silica gel were also hydrolysed by this method. 0·5 ml 4N sodium hydroxide was then added and the mixture refluxed on a water-bath for 2 hr. Colorimetric determination of fatty acid levels showed that total hydrolysis had occurred by this time. The solution was acidified with 1·0 ml 4N acetic acid, then 3 ml distilled water and 5 ml chloroform were added, and the fatty acids extracted by shaking. The tube was centrifuged at *c.* 1000 r.p.m. for a few minutes to complete separation of the upper phase, which was discarded, from the lower, predominantly chloroform phase. This hydrolysate contained the fatty acids in a constant volume of 8·3 ml. In recovery experiments with [^{14}C]palmitic acid, 99·6% of fatty acids were extracted into the hydrolysate.

If a lipid extract was not required for fractionation or for radioactive assay, the fatty acid esters were hydrolysed directly. About 0·5–1·0 g of feed or faeces that had not been dried were weighed into a large Quickfit tube, then 20 ml ethanol and 2 ml 4N sodium hydroxide were added and the mixture refluxed for 2 hr. The solution was acidified with 4 ml 4N acetic acid, then 12 ml water and 20 ml chloroform were added and the fatty acids extracted by shaking. The upper phase was removed carefully with a Pasteur pipette connected to a water-pump, and the volume of the hydrolysate measured. Although this volume was always of the order of 33 ml, it varied slightly due to the addition of small, though variable, amounts of water from the sample.

Levels of fatty acids extracted by this direct method did not differ significantly from the levels obtained when the lipids were extracted with chloroform–methanol before hydrolysis.

(ii) *Bile*

To hydrolyse the fatty acid esters in bile, 0·2–0·5 ml bile was added to 5 ml ethanol and 0·5 ml 4N sodium hydroxide, and the mixture refluxed for 2 hr. When the mixture was acidified with 1 ml 4N acetic acid, and 3 ml water and 5 ml chloroform were added, the fatty acids were extracted into the hydrolysate which had a constant volume of 8·4 ml.

Estimation of Fatty Acids

This method is based on one by Duncombe (1963, 1964). To prepare the samples, either 0·2 or 0·5 ml of the hydrolysate obtained as described in the preceding section was added to chloroform in 15-ml Quickfit tubes to give a final volume of 8 ml. Standard solutions containing either 25, 50, 75, or 100 μ-equiv/l were prepared from a 10 m-equiv/l (282·5 mg/100 ml) stock solution of oleic acid by dilution in chloroform, and were dispensed in 8-ml portions into 15-ml Quickfit tubes. Blank tubes with 8 ml chloroform were also prepared.

For formation of copper soaps, 3 ml of copper reagent that contained 10 vol. 6% cupric nitrate, 9 vol. 15% triethanolamine (w/v), and 1 vol. 1N acetic acid was added to each tube and mixed with the chloroform phase by shaking manually for about 15 sec. The tubes were centrifuged at about 1000 r.p.m. for a few minutes, then the upper phase, which contained the copper reagent, was removed carefully and completely by suction.

Two portions, each of 3 ml, were removed from the lower phase, then 0·5 ml of 0·1% sodium diethyldithiocarbamate in s-butanol was added and the colour read at 440 mμ.

The optical density produced from a standard solution of oleic acid was not significantly altered by variations in the time of storage, or the concentration of the diethyldithiocarbamate (0·04–0·2%) or of the cupric nitrate (2–6·5%), or the time of shaking of copper reagent with the chloroform solution of fatty acids. The final colour was stable for some hours.

Standard curves constructed from solutions of oleic, palmitic, myristic, lauric, and capric acids revealed an apparently straight-line relationship between concentrations (20–100 μ-equiv/l) and optical density. Although the colorimetric response does vary a little between different fatty acids with 12–18 carbon atoms, these variations are minor, and are not likely to affect the estimated fatty acid content of nutritionally important materials (cf. Heath, Adams, and Morris 1964; Hilditch and Williams 1964; Miller and Rice 1967).

If the concentration of fatty acids was very low, it was necessary to take 1·0 ml of hydrolysate, instead of the 0·2 or 0·5 ml generally required. However, if 1·0 ml was used, the ethanol that it contained was sufficient to cause spuriously high values for the optical density. To overcome this, the hydrolysate was washed either once or twice with 5 ml distilled water before the 1·0 ml was removed. This washing removed most of the ethanol, and also reduced the volume of the hydrolysate to either 5·2 or 5·0 ml, depending on whether one or two washings were used.

The extent of interference by other contaminants including pigments was determined by comparing the optical density produced by direct estimation of the fatty acids with the value obtained after purification of these acids on thin-layer

chromatograms. In other series of tests, extracts of feed materials were applied to thin-layer plates, and the lipid fractions that were separated were hydrolysed and the fatty acids extracted and measured. In each series, no significant differences existed between the values obtained after purification and those obtained by direct estimation of the fatty acids. Hence, normal contaminants do not interfere with the estimation of total fatty acids by the method described.