adrenocorticotrophic hormone and corticosteroids cause an efflux of ascorbic acid from adrenal-cortex slices *in vitro*, this being due to inhibition of active transport into the tissue.

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Metabolism of Palmitate in Sheep

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The metabolic importance of the plasma free fatty acid fraction of circulating lipids, first suggested by the observations of Dole (1956) and Gordon & Cherkas (1956), has been confirmed by measurements of turnover rate based on isotope dilution in man (Laurell, 1957; Frederickson & Gordon, 1958; Havel, 1961), dogs (Bierman, Schwartz & Dole, 1957; Armstrong *et al.* 1961) and rats (Olivecrona, 1962). Single-injection studies with [14 C]palmitate or [14 C]oleate have formed the basis of most investigations, but Armstrong *et al.* (1961) measured turnover of free fatty acid in dogs by a continuous-infusion technique. In this investigation, as in most others, plasma free fatty acid was treated as a single metabolic fraction and the assumption made that the metabolism of the injected fatty acid (palmitate) was representative of that of the whole plasma free fatty acid fraction. We have measured entry rates of palmitate in sheep by isotope-dilution procedures based on the continuous infusion of [14 C]palmitate. This acid was isolated from the other components of plasma free fatty acid by chromatography before measurement

of specific radioactivity. Comparison of the specific radioactivities of plasma palmitate and blood carbon dioxide allowed the contribution of palmitate to total carbon dioxide production to be estimated roughly. The transfer of radioactivity from palmitate into other plasma lipids and certain blood constituents was also followed.

MATERIALS AND METHODS

Animals. Merino ewes and wethers (age 2–3 years) were housed indoors and fed with lucerne chaff (800 g./day). The animals were trained to stand quietly in stocks, sheep failing to respond to training (about 50%) being rejected.

Measurement of rate of entry of palmitate. The general procedure described by Annison & White (1961) for the measurement of glucose entry rates was used. Labelled palmitate in albumin (0.75%) was infused at 1.0 μ C, 0.05 μ mole/min., without using a priming dose (Armstrong *et al.* 1961). Constancy of plasma FFA* concentration is a prerequisite for entry rate measurements, but FFA concentrations in sheep rise sharply in response even to mild excitement. Disturbance of the animal due to handling or

* Abbreviation: FFA, free fatty acid.

sampling procedures before and during experiments was avoided as far as possible. Animals were not fed on the day of an experiment.

Aqueous solutions of sodium [$1\text{-}^{14}\text{C}$]palmitate and uniformly labelled sodium [^{14}C]palmitate (obtained from The Radiochemical Centre, Amersham, Bucks.) in albumin were prepared by the method of Laurell (1957). The albumin (human) was obtained from Boehringer und Soehne G.m.b.H., Mannheim, W. Germany (*reinst* grade, containing not more than 0.03% of α -globulin, 0.015% of β -globulin and 0.03% of γ -globulin determined by immunological assay).

Blood samples (30 ml.) were immediately centrifuged at 8000g for 15 min. to remove cells and the plasma was extracted at once with the appropriate solvent mixture. Heparin was used as an anticoagulant.

Extraction and fractionation of lipids. Plasma was extracted with 20 vol. of chloroform-methanol (2:1, v/v) according to the method of Folch, Lees & Sloane-Stanley (1957), and the main lipid classes (cholesterol esters, cholesterol, triglycerides, FFA and phospholipids) were separated by silicic acid chromatography by the procedure of Hirsch & Ahrens (1958). FFA accompanied the triglyceride fraction and was isolated by partitioning the mixture between light petroleum (b.p. 60–80°) and N-NaOH (Bergström, 1952).

The FFA fraction of plasma lipids was on some occasions isolated by the method of McCarthy & Duthie (1962), which separates lipid mixtures into neutral lipids, FFA and phospholipids.

Analysis of fatty acids. The proportions of the individual long-chain fatty acids in plasma FFA were determined by gas-liquid chromatography of the methyl esters at 180°, with 120 cm. glass columns (int. diam. 4 mm.) packed with kieselguhr (Gas-Chrom P, 60–80 mesh, obtained from Applied Science Laboratories, State College, Pa., U.S.A.) coated with 10% Apiezon L high-vacuum grease (Metropolitan-Vickers Electrical Co. Ltd., U.K.). Acid concentrations were measured with a β -ionization detector (W. G. Pye and Co. Ltd., Cambridge). Fatty acids were methylated with 12.5% (w/v) boron trifluoride in methanol by the method of Metcalfe & Schmitz (1961), adapted for use with smaller amounts of fatty acids. Fatty acid mixture (1 mg.) and 1.0 ml. of reagent were heated in a 10 ml. glass-stoppered tube at 100° for 2 min. After cooling to room temperature, 5 ml. of light petroleum (b.p. 40–60°) and 4 ml. of water were added, the mixture was shaken for 30 sec. and allowed to separate into two phases. The light petroleum was removed by heating at 40° in a stream of N_2 and 0.1 μl . samples of the esters were examined by gas-liquid chromatography.

Isolation of fatty acids by reverse-phase partition chromatography. The method of Howard & Martin (1950) was used. Kieselguhr (Hyflo Super-Cel, Johns-Manville and Co.) was size-graded and treated with dichlorodimethylsilane as described by Lipsky, Haavik, Hopper & McDivitt (1957). Fatty acids (1–10 mg.) were separated on columns containing 9 g. of stationary phase in water-jacketed glass columns (1 cm. diam.) maintained at 37°. Eluate was collected in 2 ml. fractions and titrated with 5 mM-KOH in a stream of N_2 with 0.1 ml. of 0.02% (w/v) bromothymol blue as indicator. Palmitate and oleate were eluted together as a single band with aq. 65% (v/v) acetone; stearate was eluted with aq. 70% (v/v) acetone.

When necessary, oleate was removed from palmitate by oxidation of the oleate with cold alkaline permanganate (Crombie, Comber & Boatman, 1955), with modifications for use with smaller quantities of fatty acids. The palmitic acid fraction recovered was examined for purity by gas-liquid chromatography; if it contained unchanged oleic acid the oxidation procedure was repeated.

When it was necessary to isolate and determine the specific radioactivity of oleate in the presence of palmitate, the mixture of the acids was isolated by reverse-phase chromatography, and the oleate dihydroxylated by treatment of the mixture with H_2O_2 in ethyl formate and formic acid (Bergström & Pääbo, 1954). When the mixture was chromatographed by the reverse-phase system, 9,10-dihydroxystearic acid was eluted with aq. 45% (v/v) acetone, palmitic acid requiring aq. 65% (v/v) acetone.

Estimation of cholesterol, glycerides and phosphorus. The method of Abell, Levy, Brodie & Kendall (1952) was used for hydrolysis and estimation of cholesterol, with a modified Lieberman-Burchard reagent described by Huang, Chen, Wefer & Raftery (1961). Glycerides were measured by the method of Carlson & Wadstrom (1959) and phosphorus by the method of Allen (1940).

Specific radioactivities of blood acetate, β -hydroxybutyrate and carbon dioxide. Acetate was estimated as described by Annison & White (1962). β -Hydroxybutyrate was isolated from blood by column chromatography on silicic acid, and the specific radioactivity determined (Leng & Annison, 1963). Blood CO_2 was measured as described by Annison, Scott & Waites (1963).

Plasma free fatty acid. Plasma FFA was measured by the method of Dole (1956) with modifications suggested by Dr D. B. Lindsay (Annison, 1960).

Specific radioactivity of lipid fractions. Isolated lipid fractions were either oxidized by the procedure of Van Slyke & Folch (1940), and assayed as BaCO_3 (Annison & White, 1961), or counted in a liquid-scintillation counter (Nuclear Enterprises, Edinburgh, Assembly NE 8301) in toluene solution containing 0.4% of 2,5-diphenyloxazole and 0.01% of 1,4-bis-(5-phenyloxazol-2-yl)benzene.

RESULTS

Measurement of entry rates of palmitate. Entry rates of palmitate were determined on seven wethers and three ewes, which were starved for 24 hr. before infusion. The mean value for palmitate entry was 0.60 mg./min./kg. (range 0.25–1.03). A wide range of plasma palmitate concentrations was encountered (mean value 0.109 m-moles/l., range 0.026–0.248), which roughly reflected plasma FFA concentrations, the palmitate content of FFA varying from 20.0 to 29.3% (mean value 24.2%). Some dependence of palmitate entry rate on plasma palmitate concentrations was observed (Fig. 1). When the results were expressed in terms of total plasma FFA, the plot of FFA entry rate against FFA concentration was similar to the plots of palmitate entry against palmitate concentration or FFA concentration (the three plots showed $0.05 > P > 0.01$ for the null hypothesis that there

is no dependence of entry rate on concentration). If it is assumed that 4 % of the body weight of sheep is plasma (Schambye, 1952) and that the FFA pool occupies this space, the turnover time was about 2.0 min. in the present studies (cf. Armstrong *et al.* 1961). Application of the mathematical relationships employed by Armstrong *et al.* (1961) in their studies on FFA turnover in dogs showed that plasma palmitate specific activity during the infusion of [14 C]palmitate was independent of the specific activity existing 30 min. previously.

Individual free fatty acids in sheep plasma. Gas-liquid-chromatographic analysis of plasma FFA from ten sheep showed that the total of the palmitic acid and the C_{18} acids accounted for about 80 % of total FFA (Table 1).

Incorporation of labelled palmitate into plasma lipids. Mean values for the concentrations of the major plasma lipids were: cholesterol, 63 (50–

81) mg./ml. (70–72 % esterified); triglyceride, 2.1 (1.7–3.0) m-equiv./l.; phospholipid, 92 (80–106) mg. of phosphorus/100 ml. The time-course of the incorporation of radioactivity into plasma lipids during the infusion of labelled palmitate is shown in Fig. 2. The specific activities of certain lipid fractions as percentages of the specific activities of plasma palmitate after infusion for 80 min. are shown in Table 2, together with the proportion of the total activity in plasma lipids accounted for by each fraction.

The transfer of radioactivity from uniformly labelled palmitate to oleate and stearate was appreciable (Table 2). Comparison of the specific radioactivities of these acids after the infusion (1.0 μ C/min.) of uniformly labelled palmitate for 80 min. suggested that at least 7 % of both oleate and stearate were derived from palmitate.

Oxidation of plasma palmitate. The specific activity of venous (jugular) blood carbon dioxide during the infusion is shown in Fig. 3. The slope of the curve after 180 min. was somewhat greater than that observed at comparable times during the intravenous infusion of bicarbonate into sheep (Annison *et al.* 1963). The continued slow rise of specific radioactivity of blood carbon dioxide with time probably reflected the extensive transfer of radioactivity from plasma palmitate to other lipid fractions (Table 2) in addition to the non-attainment of complete equilibrium between blood carbon dioxide and certain body carbon dioxide pools. Rough estimates of the contribution of plasma

Fig. 1. Relationship of palmitate entry rate to plasma palmitate concentration. The regression line corresponding to the equation $y = 2.9416x + 0.2824$ showed a correlation coefficient 0.722, $0.05 > P > 0.01$.

Table 1. Composition of plasma free fatty acid from ten sheep

Fatty acid	Mean value (range) (moles/100 moles)
Below C_{10}	1.5 (0.7–4.1)
11:0	0.3 (0.1–0.5)
12:0	0.6 (0.1–1.1)
13:0	0.3 (0.1–0.6)
14:0	2.8 (0.9–5.6)
15:0	2.4 (1.1–6.0)
16:1	2.6 (1.4–5.0)
16:0	24.3 (15.7–40.7)
17:0	4.6 (3.3–10.8)
18:1	29.6 (16.0–41.8)
18:0	27.2 (16.6–45.3)
19:0	2.8 (1.1–9.5)

Fig. 2. Incorporation of radioactivity into plasma lipids during the infusion of [14 C]palmitate at 1.0 μ C, 0.05 m-mole/min. Δ , Free cholesterol; $\square$, lecithin; $\circ$, triglyceride; $\blacktriangle$, sphingomyelin; $\bullet$, cholesterol esters. The arrow indicates the end of the infusion.

Fig. 3. Increase in specific activity of venous blood CO_2 during the infusion of $[\text{U-}^{14}\text{C}]$ palmitate at $0.35 \mu\text{C}$, 0.05 m-mole/min . The arrow indicates the end of the infusion.

Fig. 4. Relationship between plasma palmitate concentration and the contribution of palmitate to total CO_2 production.

Table 2. Incorporation into plasma lipid of $[\text{1-}^{14}\text{C}]$ - and $[\text{U-}^{14}\text{C}]$ -palmitate when infused at $1.0 \mu\text{C/min}$. for 80 min.

Lipid fraction	A, $[\text{1-}^{14}\text{C}]$ Palmitate; B, $[\text{U-}^{14}\text{C}]$ palmitate.			
	Sp. radioactivity (% of sp. activity of total plasma palmitic acid)		Fraction activity (% of total activity in plasma lipids)	
	A	B	A	B
Triglyceride	0.48	1.60	4.38	11.45
Cholesterol	1.80	7.65	3.18	9.86
Cholesterol esters	0.16	2.19	0.77	8.20
Lecithin	0.41	2.73	2.44	12.66
Sphingomyelin	0.12	0.25	0.21	0.38
Acetic acid	2.18	1.77	0.31	0.46
β -Hydroxy-butyrate	2.00	23.3	0.24	5.89
Stearate	9.64	7.45	11.94	5.31
Oleate	8.73	7.14	11.31	2.92

palmitate to total production of carbon dioxide were obtained by comparing the specific radioactivities of plasma palmitate and blood carbon dioxide after the infusion of labelled palmitate for 240 min. In four experiments values 2.8, 4.7, 5.3 and 10.2 % were obtained, and a linear relationship was observed when these figures were plotted against the corresponding concentrations of plasma palmitate (Fig. 4).

Measurements of arterial (carotid) and venous (jugular) blood carbon dioxide during the continuous infusion of $[\text{14C}]$ palmitate (two experiments) showed no detectable differences in specific radioactivity, suggesting that palmitate is oxidized by the tissues of the head to the same extent as in the whole body.

DISCUSSION

The mobilization of fatty acids from adipose-tissue triglycerides, which constitute the major source of oxidizable substrate in mammals, occurs exclusively in the form of FFA. The rate of entry of FFA into plasma is therefore a valuable parameter of fat metabolism in the intact animal, and when information on FFA oxidation is also available the role of FFA in overall metabolism can be quantitatively assessed. The present studies have established the importance of palmitate as an energy source in sheep, this single FFA apparently accounting for about 5 % of total production of carbon dioxide in resting, starved animals. Palmitate accounts for about 20 % of plasma FFA in sheep (Table 1), and if palmitate metabolism is representative of that of plasma FFA the contribution of FFA to total production of carbon dioxide was about 25 %. Armstrong *et al.* (1961) reported corresponding values of 21–31 % for the dog.

The dependence of palmitate entry rate on plasma concentration (Fig. 1) was in agreement with results obtained in dogs by Armstrong *et al.* (1961), who infused $[\text{14C}]$ palmitate but measured only the entry rate of total plasma FFA. When the results of the present studies were expressed in terms of total plasma FFA, the regression obtained when FFA entry rate was plotted against FFA concentration was similar to those obtained when palmitate entry was plotted against palmitate concentration or FFA concentration, but of a lower level of significance than the regression of the plot of FFA entry rate against FFA concentration reported by

Armstrong *et al.* (1961). The present results with sheep provide general support for the concept that the rate of production of FFA controls the concentration of FFA, which in turn controls rate of uptake (Armstrong *et al.* 1961), but also suggest that factors other than FFA concentration play a part in the control of FFA mobilization and uptake. The rate of uptake of FFA by liver *in vivo* has been shown to depend on the concentration of plasma FFA (Fine & Williams, 1960; McElroy, Seifert & Spitzer, 1960).

There is good evidence from studies *in vitro* that the oxidation of palmitate by skeletal muscle, diaphragm and liver is roughly proportional to the concentration of FFA in the medium (Fritz, Davis, Holtrop & Dundee, 1958; Eaton & Steinberg, 1961). Several groups of investigators (see review by Steinberg, 1963) have observed that the oxidation of injected labelled FFA is increased when circulating FFA is raised, e.g. in starvation, or by the infusion of adrenaline, and decreased when plasma FFA is reduced by glucose feeding. In the present studies the rate of oxidation of palmitate in the intact sheep was shown to be closely dependent on plasma concentration (Fig. 4). The rapid transfer of radioactivity from palmitate to other lipid fractions (Table 2) emphasizes that values obtained for the contribution of palmitate to overall oxidative metabolism include the results of both direct and indirect (via metabolic intermediates) oxidation.

The rapid incorporation of labelled palmitate into plasma glycerides (Fig. 2) suggested that plasma FFA was a major source of the fatty acids of plasma triglycerides, in agreement with earlier observations by Havel (1961) in man. The relatively rapid transfer of radioactivity into cholesterol indicated that acetyl-CoA arising from fatty acid degradation is available for cholesterol synthesis. The low specific radioactivity of cholesterol esters relative to cholesterol suggested that in the sheep, as in man (Riley & Nunn, 1960) and the cow (Lough & Garton, 1957), unsaturated fatty acids are preferentially esterified by cholesterol. Similarly, the ready incorporation of palmitate into lecithin is in agreement with the observations of Hanahan & Blomstrand (1956), who showed that palmitate was incorporated into lecithin more rapidly than oleate.

SUMMARY

1. Entry rates of palmitate were measured in ten starved (24 hr.) sheep (seven wethers, three ewes).

2. Plasma palmitate concentrations, which roughly paralleled plasma concentrations of free fatty acid, varied from 0.026 to 0.248 m-mole/l., mean 0.109; the palmitate content of plasma free fatty acid varied from 20.0 to 29.3%, mean 24.2%.

3. Some dependence of palmitate entry rate on plasma concentration was observed, but the results suggested that factors other than concentration of free fatty acid are involved in the control of mobilization and uptake of free fatty acid.

4. Considerable incorporation of radioactivity into blood cholesterol (free and esterified), phospholipid, triglyceride, acetate, β -hydroxybutyrate and free stearate and oleate occurred during the infusion of [^{14}C]palmitate.

5. A linear relationship was found between the contribution of palmitate to production of carbon dioxide and the concentration of plasma palmitate.

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The Isolation and Specific Activity of Rabbit-Muscle Glyceraldehyde Phosphate Dehydrogenase

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Reported values for the specific activity of glyceraldehyde phosphate dehydrogenase [D-glyceraldehyde 3-phosphate-NAD oxidoreductase (phosphorylating), EC 1.2.1.12] isolated from rabbit skeletal muscle have varied considerably (Table 1). The present investigation was undertaken in an attempt to obtain material of reproducibly high specific activity. The approach to a quick and reproducible method of isolating the enzyme was based on an observation of Smithies (1954). He showed that the composition of a crystalline sample of β -lactoglobulin (containing two or possibly three components) remained unchanged throughout repeated crystallizations owing to the formation of highly stable mixed crystals. He concluded that '... crystallisation should be avoided in the further purification of proteins which have already been crystallised once'.

Recent studies suggest that the enzyme is composed of four sub-units, each of molecular weight about 35 000, giving a total molecular weight of about 140 000 (Dandliker & Fox, 1955; Fox & Dandliker, 1956; Elias, Garbe & Lamprecht, 1960; Harris, Meriwether & Park, 1963; Deal, 1963; Harris & Perham, 1963). A value of 140 000 has been assumed throughout this work. The zinc content and bound NAD content of the enzyme were investigated, since values for these entities have also been in some doubt.

MATERIALS

Water from an all-Pyrex still was used throughout. Conc. NH_3 (approx. 8N) and conc. HCl (approx. 9N) were made by isopiestic distillation (Irving & Cox, 1958) and

stored in Pyrex bottles, except where otherwise stated. L-Cysteine hydrochloride was the laboratory reagent from British Drug Houses Ltd., Poole, Dorset. Dichlorodimethylsilane, $\text{Cl}_2\text{Si}(\text{CH}_3)_2$ (laboratory reagent from British Drug Houses Ltd.), was stored at 0–3°. Dithizone (analytical reagent from British Drug Houses Ltd.) was purified by the method of Andrew (1946). EDTA (disodium salt) (laboratory reagent from British Drug Houses Ltd.) was recrystallized from boiling water. DL-Glyceraldehyde 3-phosphate was obtained as the barium salt of the diethylacetal from the Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., and was stored at 0–3°; solutions were prepared by the method of Racker, Klybas & Schramm (1959). A sample of glyceraldehyde phosphate dehydrogenase was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, W. F. Germany; all other samples were prepared as described

Table 1. *Specific activity of various crystalline preparations of glyceraldehyde phosphate dehydrogenase*

Values are calculated from the initial rate of reaction in the presence of excess of D-glyceraldehyde 3-phosphate, NAD and Na_2HAsO_4 .

Reference	pH	Temp.	Specific activity (μ moles of NAD/min./mg.)
Cori, Slein & Cori (1948)	8.6	27°	67
Beisenherz <i>et al.</i> (1953)	8.7	—	41
	7.5	—	24
Koeppel, Boyer & Stulberg (1956)	8.6	27	158*†
Astrachan, Colowick & Kaplan (1957)	8.5	28	54 (average)†
	8.5	28	88 (best)†
Czok & Bücher (1960)	—	25	92

* Na_2HAsO_4 was replaced by Na_2HPO_4 .

† Calculated from the authors' values, which were given in moles of NAD/min./mole, assuming that they used the then widely accepted molecular weight of 120 000 (Taylor, 1951; Taylor & Lowrey, 1956).

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