Biochem. J. (1966) **101**, 317

Utilization of Free Fatty Acids by Starved and Pregnant Sheep

BY W. M. F. LEAT AND E. J. H. FORD*
Agricultural Research Council, Institute of Animal Physiology, Babraham, Cambridge

(*Received* 25 *February* 1966)

1. The rate of entry into the plasma of stearic acid in fed and starved non-pregnant sheep and of palmitic acid in fed and starved pregnant sheep has been measured by a continuous-infusion isotope-dilution method. 2. In non-pregnant sheep the entry rate of stearic acid rose from 0·38mg./min./kg. when fed to 0·69mg./min./kg. after 72hr. starvation. In pregnant sheep, the entry rate of palmitic acid rose from 0·55mg./min./kg. when fed to 0·64mg./min./kg. on starvation. 3. The entry rates of palmitic acid and stearic acid are related to their respective plasma concentrations. 4. At a given plasma concentration the entry rate of palmitic acid in pregnant sheep was greater than that of stearic acid in non-pregnant sheep. 5. There was no detectable conversion of palmitate or stearate into other plasma long-chain fatty acids. There was negligible incorporation of fatty acids into other plasma lipids with the exception of the plasma triglycerides of fed pregnant sheep. 6. Up to 12% of expired carbon dioxide was derived from palmitic acid or stearic acid. The high rate of oxidation of plasma palmitic acid in fed pregnant sheep is noteworthy.

A number of investigators have used continuous-infusion isotope-dilution methods to measure the rate of utilization of metabolites by sheep. For example, the utilization of glucose has been studied by Annison & White (1961), Bergman (1963) and Ford (1963) and the utilization of acetate has been studied by Annison & Lindsay (1961), Lindsay & Ford (1964) and Sabine & Johnson (1964).

Armstrong *et al.* (1961) used the method to study the turnover of FFA† by the dog, but no information was available on ruminants when the present investigation was begun. Since then West & Annison (1964) have published information on the metabolism of palmitate in non-pregnant sheep starved for 24hr.

The aim of the present experiments was to study the utilization of the FFA fraction of the plasma lipids in fed and starved pregnant and non-pregnant sheep. ^{14}C-labelled stearic acid and palmitic acid were used and information was obtained on the metabolism of the individual acids as well as the total FFA fraction of the plasma lipids.

MATERIALS AND METHODS

Animals. These were Clun Forest ewes from the Institute flock. They were fed on pasture supplemented with hay and a concentrate mixture at a rate sufficient to produce a small but steady increase in body weight. The pregnant animals had been served by a ram on known dates and conception was confirmed by X-ray examination towards the end of pregnancy. All the animals were trained to mount a ramp and stand in the stocks where the perfusions were to be carried out. Animals to be starved were taken into the animal house and confined in pens for 72hr. without food but with ample water.

Technique of infusion and collection of samples. Before an infusion was carried out polythene cannulae were inserted, after the injection of a local anaesthetic, in a downward direction into both external jugular veins. To avoid contamination of blood samples by the infusion fluid, the infusion cannula was inserted about 12in. into one jugular vein and the cannula for withdrawal of blood samples was inserted about 6in. into the opposite jugular vein. The ewe was then placed in the stocks and 100ml. of blood was withdrawn from the sampling cannula into 3ml. of saturated citrate solution as anticoagulant. The plasma was separated by centrifugation at 4°.

The infusion fluid was prepared by adding to 50ml. of plasma 100μc of fatty acid, as the sodium salt, and 0·9% NaCl solution to give a total volume of 220–260ml. for infusion. In the four experiments with pregnant animals [U-^{14}C]palmitate (365μc/mg.) was used and in the other four experiments with non-pregnant sheep [U-^{14}C]stearate (350μc/mg.) was used. Both these materials were obtained from The Radiochemical Centre, Amersham, Bucks. The solution of radioactive fatty acid in diluted plasma was infused into the long catheter at a constant rate for about 130min. with a D.C.L. micro-pump (The Distillers Co. Ltd., Great Burgh, Epsom, Surrey).

Blood samples (about 20–25ml.) were collected at intervals of about 10min. during the last 60min. of the infusion into chilled tubes containing a dry mixture (3:2,

* Present address: Department of Veterinary Clinical Studies, University of Liverpool Veterinary Field Station, 'Leahurst', Neston, Cheshire.

† Abbreviation: FFA, free fatty acids.

w/w) of ammonium oxalate and potassium oxalate (20mg./10ml. of blood) and stored in crushed ice until centrifuged at 4° to obtain the plasma.

During this period the animal's head was enclosed in a polythene box with entry and exit valves for air. Expired air passed via a flow-meter to a spirometer from which a constant flow was taken through a drying tower containing $Mg(ClO_4)_2$, through a Katharometer (Cambridge Instrument Co., Cambridge) and then into a portable gas monitor (model 53C; Electronic Instruments Ltd., Richmond, Surrey). The gas monitor consisted of a 2l. ionization chamber and an electrometer, the current from which was recorded on a Honeywell–Brown chart recorder. The output of the Katharometer gave a tracing of CO_2 concentration on a Cambridge recorder calibrated for 0–5% CO_2 in expired air.

Chemical methods and assay of radioactivity. (*a*) Plasma glucose. A sample of plasma was deproteinized with 0·3N-$Ba(OH)_2$ and 5% (w/v) $ZnSO_4,7H_2O$. Glucose in the filtrate was determined by the enzymic method of Huggett & Nixon (1957).

(*b*) Plasma ketone bodies. Plasma ketone bodies in the deproteinized filtrate were measured by a slight modification of the method of Bakker & White (1957).

(*c*) Plasma free fatty acids. A 5ml. sample of plasma was extracted by a modification of Dole's method (Gordon, 1960) by using proportional increases in the volumes of reagents. From the resulting heptane supernatant, duplicate 3ml. portions were titrated with 0·02N-NaOH and a 10ml. portion was evaporated almost to dryness. This residue was transferred quantitatively to a thin-layer chromatography plate (10cm.×20cm.) coated with silica gel G and the neutral lipids were separated by developing in light petroleum–diethyl ether–acetic acid (90:10:1, by vol.) (Bowyer, Leat, Howard & Gresham, 1963). The radioactivity of the plasma lipids separated by thin-layer chromatography was assayed basically as described by Brown & Johnston (1962). The lipid areas, after location with 2,7-dichlorofluorescein, were scraped from the plates and transferred quantitatively to scintillation vials. Then 1ml. of liquid scintillator (phosphor type D.E.M.; Panax Ltd., Redhill, Surrey) was added and the radioactivity assayed in a liquid-scintillation counter (Panax Equipment Ltd.).

A 1ml. portion of suitably diluted infusion mixture was mixed with 4ml. of plasma and the FFA fraction was separated and assayed for radioactivity as described above with about 45% efficiency.

Plasma FFA separated by thin-layer chromatography on silica gel G were converted into their methyl esters as described by Bowyer *et al.* (1963). The radioactivity in the individual fatty acids was estimated by separating the methyl esters at 180° on a column containing 10% polyethylene glycol adipate on 100–120-mesh Celite, by using a Pye Radiochromatograph (W. G. Pye and Co. Ltd., Cambridge).

(*d*) Other plasma lipids. Triglycerides and phospholipids were extracted and separated as described by Nicolaysen & Nygaard (1963). Phospholipids were estimated by the method of Allen (1940) and triglycerides by that of Van Handel & Zilversmit (1957). Free and total cholesterol were isolated as their digitonides (Sperry & Webb, 1950) and then estimated as described by Leffler (1960).

(*e*) Specific radioactivities of CO_2 in expired air. These were calculated from the recordings of CO_2 content of expired air and current produced by the gas monitor after the monitor had been calibrated by using known amounts of $^{14}CO_2$.

Measurement of entry rate. When radioactive FFA of negligible carrier content is infused into the blood stream at a constant rate so that the specific radioactivity of the fraction remains constant, the rate of entry of FFA from the tissues into the plasma (measured in mg./min.) is given by the rate of infusion of radioactivity (counts/100sec./min.) divided by the specific radioactivity of FFA in plasma (counts/100sec./mg.). Provided that the concentration of FFA in plasma is constant the entry rate is equal to the removal rate from the plasma, i.e. the transfer or utilization rate.

Calculation of turnover time. If it is assumed that 4% of

Table 1. *Particulars of animals used in fatty acid utilization studies*

Results are given as the means ±S.E.M., with numbers of determinations in parentheses.

Sheep no.	Wt. (kg.)	Nutritional status	Reproductive status	Plasma concn. Glucose (mg./100ml.)	Ketone bodies (mg./100ml.)	FFA (μmoles/l.)	Weight change in 3 days before experiment (kg.)
L166	62	Fed	Non-pregnant	85·0±0·5 (3)	2·1±0·26 (3)	536±13 (6)	+0·5
L166	53	Starved 3 days	Non-pregnant	58·7±2·4 (3)	7·6±0·80 (3)	1516±75 (6)	−10
L78	57	Fed	Non-pregnant	80·3±1·4 (3)	2·1±0·30 (3)	406±16 (6)	0
L78	49	Starved 3 days	Non-pregnant	56·3±2·8 (3)	5·4±0·25 (3)	1058±44 (6)	−6
K92	70	Fed	Pregnant (twins) for 111 days	86·6±6·5 (3)	3·5±1·70 (3)	932±49 (6)	+1
K92	69	Starved 3 days	Pregnant (twins) for 123 days	59·7±1·7 (4)	6·2±1·50 (4)	1054±61 (6)	−5
J72	74	Fed	Pregnant (single) for 112 days	130·7±4·1 (4)	4·7±0·14 (7)	491±22 (6)	0
J72	69	Starved 3 days	Pregnant (single) for 125 days	54·7±2·7 (3)	9·9±0·15 (3)	1508±35 (6)	−4·5

Table 2. *Entry rates of FFA in pregnant and non-pregnant sheep*

Sheep no.	Nutritional and reproductive status	$10^{-3}\times$ Infusion rate (counts/100sec./min.)	Sp. activity of plasma FFA (counts/100sec./mg.) Mean	Range	No. of determinations	FFA entry rate (mg./min.)	(mg./min./kg. body wt.)
L166	Fed, non-pregnant	1285	12160	10900–13000	(5)	106	1·7
L166	Starved, non-pregnant	1282	7500	5600–9900	(6)	171	3·2
L78	Fed, non-pregnant	1040	16000	15800–16200	(4)	62	1·1
L78	Starved, non-pregnant	1190	12700	9000–14400	(6)	94	1·9
K92	Fed, pregnant	1240	5480	4600–6800	(6)	226	3·2
K92	Starved, pregnant	1380	5700	4600–7500	(6)	242	3·5
J72	Fed, pregnant	1380	7950	7200–9800	(6)	174	2·3
J72	Starved, pregnant	1390	4700	4100–5500	(6)	298	4·3

the body weight of the sheep is plasma and that the FFA occupy this pool, then the turnover time of palmitic acid, for example, is calculated as the time taken for as much new palmitic acid to be added to plasma as the original palmitic acid content.

RESULTS

Table 1 gives particulars of the animals used, their reproductive status and the effect of starvation on body weight and on the concentration of glucose, ketone bodies and FFA in plasma. In both pregnant and non-pregnant animals starvation was followed by a loss of weight, a fall in the concentration of plasma glucose and an increase in the concentration of FFA and ketone bodies in plasma.

The change in glucose and ketone-body concentrations in the pregnant animals was much less than had been observed in earlier experiments in which pregnant sheep were starved for 96hr. and in which symptoms of pregnancy toxaemia were produced (Ford, 1963; Ford & Lindsay, 1964).

Table 2 gives the FFA entry rates. The mean rate for non-pregnant animals of 1·4mg./min./kg. rose to 2·6mg./min./kg. after 72hr. starvation. The mean rate of 2·8mg./min./kg. for pregnant sheep rose to 3·9mg./min./kg. after the same period of starvation.

In Fig. 1 the relationship of entry rate of fatty acid to plasma concentration is demonstrated and in Table 3 entry (or utilization) rates of individual fatty acids are given. In non-pregnant sheep the entry rate of stearic acid rose from a mean of 0·38mg./min./kg. when fed to a mean of 0·69mg./min./kg. after 72hr. starvation. In the pregnant sheep, however, the effect of starvation was not as pronounced as in non-pregnant sheep, the mean entry rate of palmitic acid increasing from 0·55 to 0·64mg./min./kg.

In non-pregnant sheep between 97 and 100% of

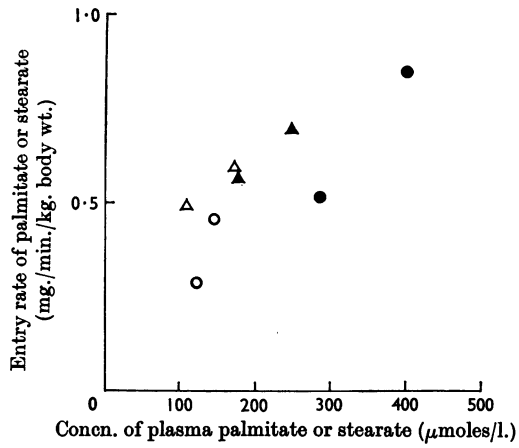

Fig. 1. Relationship of entry rate of palmitate (△, ▲) and stearate (○, ●) to mean plasma concentration in fed (△) and starved (▲) pregnant sheep and in fed (○) and starved (●) non-pregnant sheep.

the total counts in the Dole extract were in the FFA fraction but in the two fed pregnant animals 7·8 and 22% of the total counts were in the triglycerides. These percentages fell to 2·2 and 4·1% respectively in the starved states.

Fig. 2 depicts the incorporation of label into the plasma lipids of a pregnant sheep, J72, when in a fed state. In this sheep, although 22% of the total radioactivity appeared in the plasma triglycerides after 110min. of infusion, negligible radioactivity was present in the plasma cholesterol and cholesterol esters, indicating little if any conversion of long-chain fatty acids into sterols. The radioactivity in the phospholipid fraction was not estimated since the method of extraction is not quantitative for phospholipids. Fig. 2 also confirms that the specific activity of FFA in plasma was

Table 3. *Entry rates of individual FFA in starved and fed sheep*

Sheep no.	Nutritional and reproductive status	Percentage of palmitic acid or stearic acid in FFA fraction	Concn. of palmitic acid or stearic acid in plasma (μmoles/l.)	Entry rate of palmitic acid or stearic acid (mg./min./kg. body wt.)	Turnover time of plasma palmitic acid or stearic acid (min.)
Stearic acid					
L166	Fed, non-pregnant	27·0	144	0·46	3·6
L166	Starved, non-pregnant	26·4	400	0·85	5·3
L78	Fed, non-pregnant	26·6	112	0·29	4·5
L78	Starved, non-pregnant	27·0	286	0·52	6·3
Palmitic acid					
K92	Fed, pregnant	18·5	172	0·60	2·9
K92	Starved, pregnant	16·3	175	0·57	3·2
J72	Fed, pregnant	21·6	106	0·50	2·1
J72	Starved, pregnant	16·4	243	0·70	3·5

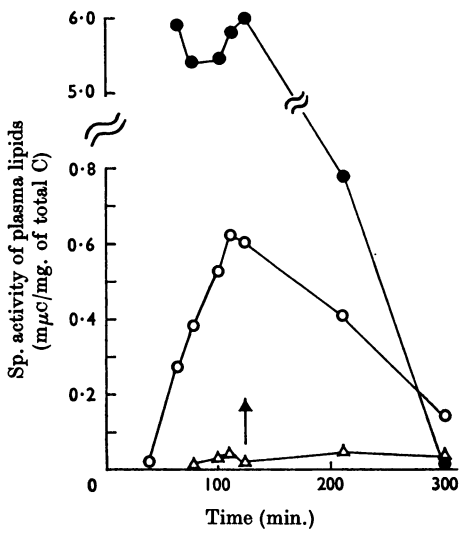

Fig. 2. Incorporation of radioactivity into the plasma lipids of a fed pregnant ewe (J 72) during and after the continuous infusion of [U-^{14}C]palmitate. ●, FFA; ○, triglycerides; △, free cholesterol and cholesterol esters. The arrow indicates the end of the infusion.

reasonably constant between 70 and 120 min. after the commencement of infusion.

When [^{14}C]palmitic acid was infused no radioactivity was detected in other long-chain fatty acids, and when [^{14}C]stearic acid was infused no radioactivity was detected in the palmitic acid. The separation between stearic acid and oleic acid on the chromatograph was not sufficiently clear-cut to conclude that no radioactivity resided in oleic acid, but if radioactivity was present in this acid it must have been extremely low.

After continous infusion of ^{14}C-labelled FFA for more than 100 min. the specific activity of the carbon dioxide in expired air of the sheep had reached a plateau level. Table 4 shows the relationship between these plateau specific activities and the specific activity of palmitate or stearate in plasma at the same time. As both specific activities are calculated on the basis of radioactivity per mg. of carbon the relation between them indicates the proportion of expired carbon dioxide that arises from the direct oxidation of palmitic acid or stearic acid (column 5). The total production of carbon dioxide has been calculated from the total volume of expired air and its carbon dioxide content (column 6). From these two values was derived the amount of carbon dioxide produced by the direct oxidation of palmitate or stearate (column 7). The values in the penultimate and last columns of Table 4 are calculated on the basis that 1 g. of fatty acid yields, on complete oxidation, 2·8 g. of carbon dioxide. The results given indicate no significant effect of pregnancy or starvation on the percentage of expired carbon dioxide arising from palmitate or stearate or on the percentage of these fatty acids oxidized to carbon dioxide. There are no values for fed non-pregnant animals because at the time of these infusions the apparatus for measuring the radioactivity of expired air was out of order.

DISCUSSION

Entry rates of FFA. West & Annison (1964) measured palmitate entry in non-pregnant sheep. The present results extend their findings and show that the entry rates of palmitate in pregnant sheep are of a similar order to those recorded by West & Annison (1964) for wethers and non-pregnant ewes. The present experiments also show that starvation for 72 hr. increases both the plasma concentration

Table 4. *Production of carbon dioxide from FFA*

Sheep no.	Nutritional and reproductive status	Mean sp. activity of plasma palmitate or stearate (mμc/mg. of C)	Mean sp. activity of expired CO_2 (mμc/mg. of C)	Percentage of CO_2 from plasma palmitate or stearate	CO_2 output (mg./min.)	CO_2 from plasma palmitate or stearate (mg./min.)	Palmitate or stearate oxidized to CO_2 (mg./min.)	Percentage of palmitate or stearate entering plasma oxidized to CO_2
L166	Starved, non-pregnant	28·4	3·0	10·5	260	27·3	9·8	21·8
L78	Starved, non-pregnant	42·3	4·2	10·0	315	31·5	11·3	43·8
K92	Fed, pregnant	22·2	2·6	11·7	365	42·7	15·5	37·0
K92	Starved, pregnant	26·2	3·1	11·8	390	46·0	16·7	42·6
J72	Fed, pregnant	27·6	2·2	8·0	520	41·6	15·2	40·8
J72	Starved, pregnant	21·5	2·4	11·1	420	46·7	17·0	35·2

and the entry rate of FFA, palmitate and stearate. The relationship of palmitate entry rate to plasma palmitate concentration has been confirmed and the results show the same correlation for stearate. Fig. 1 also shows that at a given concentration in plasma the entry rate of palmitate in pregnant sheep is greater than that of stearate in non-pregnant sheep. The calculated turnover time of 2·5 min. for plasma palmitate in pregnant fed sheep agrees well with the values found by Armstrong *et al.* (1961) for the dog and by West & Annison (1964) for non-pregnant sheep. The turnover time of plasma stearate in fed non-pregnant sheep was 4·1 min. (Table 3).

Unfortunately, the design of the experiment is such that the difference between the entry rate of palmitate in pregnant sheep and that of stearate in non-pregnant sheep cannot be explained unequivocally. This difference could be a function of the chain length of the fatty acid or of pregnancy. It is possible that the entry rates of fatty acids would increase in pregnancy since it is known that the only plasma lipid to pass through the placenta into the foetus in any appreciable amount is the FFA fraction (Van Duyne, Parker, Havel & Holm, 1960; Leat, 1966). However, the entry rates of palmitate for pregnant sheep reported in the present paper are of a similar order to values reported for non-pregnant sheep by West & Annison (1964). A more rational explanation is that the entry rate of palmitic acid is greater than that of stearic acid. The individual fatty acids of the plasma FFA fraction need not necessarily have similar turnover rates; for example, in the rat it has been shown that injected palmitic acid is removed from the plasma at a faster rate than is stearic acid (Görannson, 1965).

Incorporation of radioactivity into other plasma lipids. The transfer of radioactivity from palmitate and stearate to other long-chain fatty acids was very slight during the infusions. This is in contrast with the findings of West & Annison (1964) that at least 7% of both oleate and stearate were derived from palmitate. Differing methods of analysis may be responsible for this discrepancy and may also explain why no labelling was detected in the plasma free cholesterol and cholesterol esters in the present experiment whereas appreciable labelling of free cholesterol was reported by West & Annison (1964). Although there was considerable incorporation of palmitic acid into the plasma triglycerides of fed pregnant sheep and negligible incorporation of stearic acid into those of non-pregnant sheep it is not possible to state with any certainty whether the underlying cause is related to pregnancy or to the chain length of the fatty acid. However, pregnant sheep are known to have more stainable fat in the liver than non-pregnant animals (Ford, 1962), and this may indicate a greater production of triglyceride in the liver and subsequent release into the plasma. The liver fat content of pregnant sheep is further increased by restriction of diet (Ford, 1962) and an even greater degree of labelling of plasma triglyceride might be expected in starved than in fed pregnant sheep. In the event, incorporation of radioactivity into plasma triglycerides was lower after starvation than before. This may indicate diminished ability to transfer the synthesized triglyceride into the plasma. There was considerably greater incorporation of fatty acids into the plasma triglycerides of sheep J 72 than into those of sheep K 92. This may be merely individual variation between the sheep but it is noteworthy that sheep J 72 was

closer to parturition than was sheep K92 (−23 days compared with −50 days).

Production of carbon dioxide. In all three groups of animals from which results were available a high oxidation rate of FFA was evident. Results for individual animals indicated that up to 12% of carbon dioxide was produced by the oxidation of either palmitic acid or stearic acid. In the pregnant sheep no definite increase in the percentage contribution of palmitate to carbon dioxide production was observed when the animals were starved for 72hr. although the entry rate of palmitate did increase. The high rate of oxidation of FFA in starved pregnant and in starved non-pregnant sheep was not unexpected but that in fed pregnant sheep was rather surprising. Unfortunately, it was not possible to determine if pregnancy was a major factor since comparison with fed non-pregnant sheep could not be made owing to an apparatus failure, but these results do suggest that FFA may play an important metabolic role in pregnant sheep. West & Annison (1964) reported that for plasma concentrations of palmitate up to 100 μ-equiv./l. a linear relationship existed between plasma palmitate concentration and the percentage contribution of palmitate to carbon dioxide production. Our results indicate that for plasma palmitate and stearate concentrations greater than 100 μ-equiv./l. linearity is not maintained, and the contribution of plasma palmitate and stearate to carbon dioxide production tends to reach a plateau in the region of about 12%. This may represent an upper limit for the contribution of plasma fatty acids to carbon dioxide production.

We are grateful to Mr J. W. Clark for husbandry and preparation of the sheep and to Mr M. Rennie and Miss Judy Baker for technical assistance.

REFERENCES

Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
Annison, E. F. & Lindsay, D. B. (1961). *Biochem. J.* **78**, 777.
Annison, E. F. & White, R. R. (1961). *Biochem. J.* **80**, 162.
Armstrong, D. T., Steele, R., Altszuler, N., Dunn, A., Bishop, J. S. & de Bodo, R. C. (1961). *Amer. J. Physiol.* **201**, 9.
Bakker, N. & White, R. R. (1957). *N.Z. J. Sci. Tech.* B, **38**, 1001.
Bergman, E. N. (1963). *Amer. J. Physiol.* **204**, 147.
Bowyer, D. E., Leat, W. M. F., Howard, A. N. & Gresham, G. A. (1963). *Biochim. biophys. Acta*, **70**, 423.
Brown, J. L. & Johnston, J. M. (1962). *J. Lipid Res.* **3**, 480.
Ford, E. J. H. (1962). *J. agric. Sci.* **59**, 67.
Ford, E. J. H. (1963). *Biochem. J.* **88**, 427.
Ford, E. J. H. & Lindsay, D. B. (1964). *Res. vet. Sci.* **5**, 161.
Göransson, G. (1965). *Acta physiol. scand.* **63**, 423.
Gordon, R. S., jun. (1960). In *Lipids and the Steroid Hormones in Clinical Medicine*, p. 45. Ed. by Sunderman, F. W. & Sunderman, F. W., jun. Philadelphia: J. B. Lippincott Co.
Huggett, A. St G. & Nixon, D. A. (1957). *Lancet*, ii, 368.
Leat, W. M. F. (1966). *Biochem. J.* **98**, 598.
Leffler, H. H. (1960). In *Lipids and the Steroid Hormones in Clinical Medicine*, p. 18. Ed. by Sunderman, F. W. & Sunderman, F. W., jun. Philadelphia: J. B. Lippincott Co.
Lindsay, D. B. & Ford, E. J. H. (1964). *Biochem. J.* **90**, 24.
Nicolaysen, R. & Nygaard, A. P. (1963). *Scand. J. clin. Lab. Invest.* **15**, 79.
Sabine, J. R. & Johnson, B. C. (1964). *J. biol. Chem.* **239**, 89.
Sperry, W. M. & Webb, M. (1950). *J. biol. Chem.* **187**, 97.
Van Duyne, C. M., Parker, H. R., Havel, R. J. & Holm, L. W. (1960). *Amer. J. Physiol.* **199**, 987.
Van Handel, E. & Zilversmit, D. B. (1957). *J. Lab. clin. Med.* **50**, 152.
West, C. E. & Annison, E. F. (1964). *Biochem. J.* **92**, 573.