Letters to the Editors

Complete or incomplete breakdown of adipose tissue triglycerides in ruminants

Symonds *et al.* (1989) conclude that 'when sheep are fed on a diet from which no more than half the required metabolizable energy for late pregnancy is obtained, then lipolysis of body fat depots occurs via the incomplete breakdown of adipose tissue triglycerides'. To quote a well-known American – 'You can't be serious!'. Hormone-sensitive lipase, a protein with all the attributes of a key regulatory enzyme, catalyses the hydrolysis of triglycerides to monoglycerides, the final hydrolysis being catalysed by a monoglyceride lipase. If this latter process occurs more slowly than the initial hydrolysis, then there will be accumulation of monoglyceride either in adipocytes or elsewhere in the body (assuming transport of these molecules out of the adipocyte). There is no evidence whatsoever for either of these possibilities. A third possibility, re-esterification, is both unlikely (adipocytes do not appear to synthesize triglyceride by the monoglyceride pathway) and of course is irrelevant in the present context as it leads to no net release of fatty acid. In addition, anterio-venous difference measurement of glycerol and fatty acids across fat pads in sheep (Gooden *et al.* 1986) and non-ruminants (see Vernon & Clegg, 1985) provides no evidence for partial hydrolysis even after noradrenaline infusion.

I suggest that the problem lies in the authors' estimates of non-esterified fatty acid (NEFA) entry rates, which they themselves note are much higher than previous estimates for sheep. The authors assume that palmitic acid is representative of other fatty acids and quote several studies to support this view, but equally there are a number of studies (see Vernon, 1980) which suggest that the use of palmitic acid alone as tracer leads to a substantial overestimate of total NEFA entry rate. For example Dunshea *et al.* (1989) in the same issue, using a cocktail of different ^{14}C-labelled fatty acids, reported much lower NEFA entry rates in goats in negative energy balance. Importantly the NEFA entry rates of Dunshea *et al.* (1989) correspond reasonably well with the rate of fat loss determined in the same animals (about 65 g/d).

In contrast to the above, the NEFA entry rates reported by Symonds *et al.* (1989) are equivalent to a rate of fat (fatty acid) loss of about 1·4 kg/d in underfed sheep! This is far in excess of estimated needs of fasted sheep (100–200 g/d) (Lindsay, 1975). It is most unlikely that fatty acid re-esterification would account for much of the difference in NEFA entry rate and fatty acid requirements in such animals.

I thus conclude that the authors' claim of an important role for partial lipolysis arises from a spurious estimate of NEFA entry rate in these animals.

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Letter from Dunshea & Bell

We write this letter in response to a recent paper by Symonds *et al.* (1989). These authors simultaneously determined glycerol and non-esterified fatty acid (NEFA) kinetics in sheep during late gestation. Based on their observation that the ratio of NEFA: glycerol entry rates were greater than three (in fact mean ratios were around ten) they concluded that 'lipolysis of body fat depots occurs via incomplete breakdown of adipose tissue triglycerides'. As other researchers have failed to find ratios greater than three in a number of species and physiological states (Wilson, 1983; Dunshea & Bell, 1989; Dunshea *et al.* 1989) their data require closer examination.

There are major discrepancies between results for NEFA and glycerol specific radioactivity (SRA) in Tables 2 and 3 and calculated values for respective entry rates in Table 4. If the stated infusion rate of [1-^{14}C]palmitate and NEFA SRA's in Tables 2 and 3 are correct, then the NEFA entry rates in Table 4 are approximately 1000 fold too high. Even if this is a typographical error in the SRA values, the high NEFA: glycerol entry rate ratios still need to be explained. It is especially disturbing that the value for NEFA entry rate is approximately 1·5 kg/d, similar rates to those observed in lactating dairy cows in substantial energy deficits (Bauman *et al.* 1988). We recently demonstrated that NEFA entry rate is related to, and of a similar magnitude to, rates of fat mobilization in dairy goats (Dunshea *et al.* 1988). Clearly then, as no sheep would be able to mobilize 1·5 kg fat/d there must be a large error in the determination of NEFA entry rate. It is possible that the use of palmitate alone as a tracer for NEFA overestimates the true NEFA entry rate (Vernon, 1980). However, the estimates of NEFA entry rate obtained by Wilson (1983) and Bauman *et al.* (1988) obtained using palmitate as a tracer were close to expected and feasible rates of fat mobilization.

We therefore suggest that the high ratios of NEFA: glycerol entry rates in the study of Symonds *et al.* (1989) are due to some overestimation of NEFA entry rate rather than to incomplete breakdown of adipose tissue triglycerides.

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Reply to letters by Vernon, and Dunshea & Bell

In reply to Dr Vernon's letter, we are somewhat surprised that he finds the proposal of incomplete breakdown or partial hydrolysis of adipose tissue triglycerides during late pregnancy so ludicrous, since in a review (Vernon, 1980) he summarizes a number of studies which have described partial hydrolysis and states 'conditions under which partial hydrolysis was indicated in ruminants in vivo are those under which a very high rate of lipolysis would be expected'. Our results (Symonds *et al.* 1989) show that in twin-bearing pregnant sheep in which approximately half the daily energy requirements are supplied from fat oxidation, the ratio of non-esterified fatty acid (NEFA):glycerol entry rates is much greater than 3:1 as would be predicted from the complete hydrolysis of triglyceride. We have interpreted this evidence as suggesting that at very high lipolytic rates, hormone-sensitive lipase and perhaps lipoprotein lipase result in incomplete breakdown of triglycerides to di- and monoglycerides. We would not expect this situation to occur in non-pregnant, non-lactating sheep given short-term noradrenaline infusions (Gooden *et al.* 1986) or in well-fed lactating goats (Dunshea *et al.* 1989) since previous studies have only shown incomplete breakdown of triglycerides in ruminants in which lipolytic rate is chronically stimulated as a result of homeorrhetic adaptations to pregnancy (Bergman, 1968; Ranaweera *et al.* 1981; Symonds *et al.* 1989) or lactation (Sidu & Emery, 1973).

We do, however, share Dr Vernon's concern that the use of [^{14}C]palmitic acid as a representative tracer of other fatty acids may overestimate total fatty acid entry rate and therefore falsely indicate incomplete breakdown of adipose tissue triglyceride. There is controversy in the literature as to the accuracy of this technique (see Vernon, 1980; Dunshea *et al.* 1988; Symonds *et al.* 1989) and the only solution to this problem is to measure simultaneously total NEFA entry rate in the same animal using an equimolar mixture of ^{14}C-labelled palmitic, stearic and oleic acids together with ^{3}H-labelled palmitic acid. Until this is achieved under the same experimental conditions adopted by ourselves (Symonds *et al.* 1989), one cannot claim that the use of [1-^{14}C]palmitic acid alone gives a 'spurious estimate of NEFA entry rate'.

Dr Vernon relates the NEFA entry rates we report to fat loss but this is clearly incorrect since the NEFA must be oxidized before a true loss can occur. In fact, calculation of total fat loss from our values for NEFA oxidation yields values of 180 and 270 g/d for underfed unshorn and shorn sheep respectively, which are in agreement with published values for unshorn sheep obtained by carcass dissection (Robinson *et al.* 1978). As discussed by Dunshea *et al.* (1989), NEFA entry rate might be expected to be higher than fat oxidation rate and the weight loss of body fat since the requirement of NEFA carbons for triglyceride turnover needs to be taken into account. Incomplete breakdown of triglyceride could be accounted for by a substrate cycle involving fatty acid re-esterification of di- and monoglycerides produced by partial hydrolysis of lipoprotein triglyceride but we are not aware of any studies where this possibility has been examined. There is evidence of accumulation of di- and monoglycerides during the action of lipoprotein lipase purified from cows' milk (Nilsson-Ehle *et al.* 1973). Simultaneous uptake and release of NEFA across the hind-limb tissues of ruminants has also been observed (Symonds, 1986; Pethick *et al.* 1983; Bell & Thompson, 1979) and is consistent with the release of NEFA into the blood stream by the action of lipoprotein lipase.

The above discussion also answers the points raised in the letter from Dr Dunshea and

Professor Bell. We would also like to thank them for pointing out a typographical error in Tables 2 and 3 in which the units for NEFA SRA should be dpm/μmol rather than dpm/mmol.

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Haemoglobin regeneration efficiency

Since the use of the haemoglobin (Hb) repletion assay as proposed by Fritz *et al.* (1970) and adopted by the Association of Official Analytical Chemists (1975) as a recognized method for estimating biological availability of iron from diets, several modifications have been suggested. They include, (*a*) the use of different response criteria, e.g. packed cell volume, Hb concentration (Anderson *et al.* 1972) and Hb Fe gain (Mahoney *et al.* 1974) in calculating the bioavailability of test Fe; (*b*) the specification of the dose level of the test diet; (*c*) shortened length of the assay period (Miller, 1977); (*d*) the use of weanling rats for the assay (prophylactic) (Miller, 1982); (*e*) correction of the basal Fe in test diets; (*f*) prevention of coprophagy in the experimental animals to eliminate the over-estimation of bioavailability values (Neale, 1984).

While this assay procedure has been criticized by several workers (Amine & Hegsted, 1974; Johnson *et al.*, 1987), Mahoney & Hendricks (1982) have consistently shown that it is a reliable assay procedure for estimating available Fe from Fe salts and various food items. However, in a recent publication, Zhang *et al.* (1989) stated that an Fe complex may be a better reference material than $FeSO_4.7H_2O$ for determining the relative Fe bioavailability (RIBA) from foods. This was because they found that severe Fe depletion increased the amount of Fe absorbed from $FeSO_4.7H_2O$ added to the diet but did not increase absorption of Fe from food sources. This I think should be expected as the food sources contain some endogenous materials that would bind to and decrease the available Fe. Also

it was stated that the higher RIBA in the non-anaemic rats was due to a decreased absorption of Fe from $FeSO_4.7H_2O$ and not an increased absorption from food. The Hb regeneration efficiency of non-anaemic rats was similar regardless of whether diets contained Fe from $FeSO_4.7H_2O$ or from foods. However, Hb regeneration efficiency of anaemic rats was much higher when diets contained $FeSO_4.7H_2O$. It is obvious that the overall single factor governing the absorption of Fe in this study was the Fe status of the animals, hence the decrease in the absorption of Fe from $FeSO_4.7H_2O$ by non-anaemic rats. The absorption of Fe from a highly available Fe source (e.g. $FeSO_4.7H_2O$) could be limited as the mechanism regulating Fe absorption is very much responsive to body needs such as growth, Hb repletion and other physiological conditions.

The hypothesis of a third Fe pool needs further clarification. The mechanism of Fe absorption according to Saltman (1965) and Huebers *et al.* (1983) assumes the presence, in the lumen, of chelating agents that are capable of binding to non-haem Fe (inorganic or organic) to form complexes that are taken up by the mucosal cells of the small intestine.

Inorganic Fe could also be absorbed by direct diffusion into the mucosal cells. Is it therefore right to assume that Fe from food sources would always be presented to the mucosal cells as organic complexes? After all, the organically complexed non-haem Fe in foods, e.g. leghaemoglobin in legumes, monoferric phytate in wheat, phytoferritin in peas, conalbumin, etc., could liberate inorganic Fe^{2+}, Fe^{3+} or small-molecular-weight complexes in the lumen during digestion. What would be the overall absorption pattern of a food item of which the Fe exists as Fe^{2+}, Fe^{3+}, complexed, soluble and insoluble forms (Lee & Clydesdale, 1980)? Would it then be totally appropriate to use Fe complex as a reference material for determining RIBA of such food items?

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Reply to letter by Latunde-Dada

In response to Dr Latunde-Dada, we will explain more of our understanding of relative iron bioavailability (RIBA) and the three-Fe-pool hypothesis. We used rats of three different Fe status levels, severely anaemic, mildly anaemic and non-anaemic, which made it possible to study the pattern of Fe utilization against Fe status (Zhang *et al.* 1989). We observed that the relationship between haemoglobin regeneration efficiency (HRE) or apparent Fe absorption *v.* Fe status was different for rats given different Fe sources, i.e. $FeSO_4.7H_2O$, meat or spinach. When the Fe source was from $FeSO_4.7H_2O$, a negative relationship was seen between HRE or apparent Fe absorption *v.* Fe status. When meat was the Fe source, HRE and apparent Fe absorption showed similar patterns regardless of the animals' Fe status. Similarly, when the dietary Fe was from spinach, both HRE and apparent Fe absorption were low in severely anaemic as well as non-anaemic rats. The different patterns of Fe utilization *v.* Fe status among these Fe sources caused us to question the validity of using $FeSO_4.7H_2O$ as the reference for calculating RIBA. Since Fe status of the subjects is one of the major variants which RIBA is used to adjust for, it is important that the pattern of Fe absorption from the reference source be similar to that of food Fe.

$FeSO_4$ has been used as a reference Fe source for a long time and this is commonly accepted. However, the origin of its use was not based on careful experimentation, and no studies have been done to establish the acceptability of $FeSO_4$ as a reference Fe source. Perhaps a complexed form of Fe, such as ferric sodium EDTA which is in a complexed form in solution (Lee & Clydesdale, 1980), ferric-hydroxide polymaltose (Geisser & Müller, 1987), or ferrous ascorbate, would be a better reference Fe source. Thus, we believe more research should be done either to confirm $FeSO_4.7H_2O$ as a valid reference Fe source or to choose a more appropriate reference Fe source for RIBA determinations that parallels Fe absorption regardless of Fe status.

In support of the three-Fe-pool (haem Fe, soluble ionic Fe and complexed Fe) concept are the following: ionic Fe salts such as $FeSO_4.7H_2O$ and $FeCl_3.6H_2O$ are kept in reduced form or reduced from Fe^{3+} to Fe^{2+} in the stomach because of acidity and the presence of antioxidants such as ascorbic acid. When Fe^{2+} reaches the duodenum and before oxidation happens, this ionic Fe is diffused into the intestinal mucosal cell (Geisser & Müller, 1987). Transferrin cannot bind Fe^{2+}, so transferrin-mediated transport works only after Fe^{2+} has been oxidized to Fe^{3+} (Crichton & Charloteaux-Wauters, 1987). There is some indirect evidence which supports the possibility of a passive diffusion for $FeSO_4.7H_2O$. Fe-deficient college girls given $FeSO_4.7H_2O$ had their serum Fe absorption peak earlier than those given food Fe sources (Hendricks & Wright, unpublished results). Also, preventing coprophagy in rats reduced Fe absorption of $FeSO_4.7H_2O$ by only 8% ($P > 0.05$) but reduced Fe absorption from spinach, bran cereal or frozen green peas by about 24% ($P < 0.05$) (unpublished results).

Fe in plant foods is believed to be in complexed forms because of the many ligands in plants. It is biologically reasonable to assume that Fe exists in complexed form in plants because ionic Fe is unstable and toxic to them. We assume that part of the food Fe complexes are dissolved in the stomach and the remaining part must undergo digestion to release the Fe. The Fe^{3+} from soluble Fe complexes is believed to be combined with apotransferrin when the Fe complexes reach the duodenum as apotransferrin has a strong affinity for Fe^{3+} ions (Løvstad, 1988). On the other hand, Fe^{3+} ions in soluble Fe complexes may bind to gastrin and gastric transferrin in the stomach (Baldwin *et al.* 1986; Longano *et al.* 1988); this Fe complex is soluble at duodenal pH. The Fe is then transferred to mucosal cells by a transferrin receptor-mediated, energy-dependent transport mechanism (Huebers *et al.* 1983). It remains unclear if the two pathways exist simultaneously or if only

one exists, and if the gastric transferrin is the same one present in mucosal cells. Gastric transferrin is similar to serum transferrin in its composition except for the carbohydrate content (Baldwin *et al.* 1986).

Of course, here we are considering the main pathway of absorption of highly soluble Fe salts and food Fe complexes. In reality, a small portion of ionic Fe may be oxidized and bound to ligands to form complexes and then transported to mucosal cells through the transferrin-mediated pathway. Also, a small portion of food Fe complexes may be released, reduced to Fe^{2+} in the stomach and absorbed by the mucosal cells in the duodenum.

Haem Fe, the third Fe-pool, is believed to be taken up intact into mucosal cells as the Fe-porphyrin complex (Conrad *et al.*, 1966), but how the haem ring crosses the brush border of mucosal cells is not known.

There are still lots of mysteries and controversies as to how Fe is absorbed into the body. Some examples of the questions are: What is the food Fe profile in the stomach? How important is the subjects' Fe status on Fe absorption? With our three-pool hypothesis, we are suggesting a possible direction towards identifying mechanisms of Fe absorption. More experiments must be done before this hypothesis is confirmed or refuted. We appreciate Dr Latunde-Dada's insightful comments.

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trans-*Fatty acids and essential fatty acid metabolism in rats*

The recent paper by Hanis *et al.* (1989) raises the important question of the interaction between dietary *trans* fatty acids (*t*-FA) and essential fatty acid (EFA) metabolism in the rat. Unfortunately the authors misinterpret the presented data. On pp. 526 and 527 it is stated that 'the symptoms observed in our experiment, such as prolonged gestation period, reduced litter size and irregular oestrus cycle in the hydrogenated vegetable oil (HO)-fed dietary group are usual essential-fatty-acid-deficiency symptoms (Waltman *et al.* 1978; Parlanti & Orellana, 1985; Guesnet *et al.* 1986), *though diet HO was not essential-fatty-acid deficient*'. No evidence, either biochemical or otherwise, is presented in support of this statement. Three major questions arise out of this work: (1) Were the findings reported due to a deficiency of EFA in the diet irrespective of the *t*-FA intake? (2) Was EFA deficiency induced by *t*-FA in the diets containing marginal EFA levels? (3) Were the observations due to *t*-FA alone?

Hanis *et al.* (1989) quote the dietary level of *cis,cis*-linoleic acid (18:2) as 0·54% energy (p. 520, line 37) while in our calculations this would be a maximum of 0·2% of the diet. This level of dietary *cis,cis*-18:2 is, according to reliable sources in the literature, much lower than that required by rats, particularly for reproduction, and would thus be construed as deficient. For instance the National Research Council (1978) quote 0·6% dietary level of *cis,cis*-linoleic acid as the requirement of the laboratory rat, and Pudelkewicz *et al.* (1968) and El Madfa *et al.* (1983) quote a requirement of 1% dietary energy as linoleic acid, particularly for reproduction. Although linolenic acid (18:3$n-3$) in the HO diet (Tables 1 and 2 of Hanis *et al.* (1989)) amounted to 0·075%, the total EFA content would be a maximum of 0·275%.

Guesnet *et al.* (1986, 1988) have proposed a dietary 18:3$n-3$ concentration of 0·2% to meet minimum requirements. According to these authors diet HO would be deficient in both 18:2 and 18:3 fatty acids.

With these considerations in mind and in the absence of additional biochemical evidence, the conclusions of Hanis *et al.* (1989) that giving hydrogenated *t*-FA adversely affected litter size, sperm morphology, etc. through interference with the linoleic acid pathway are unfounded. These effects could have arisen in rats fed on diets with similar contents of both 18:2 and 18:3 fatty acids but devoid of the *t*-FA. The studies, however, appear to support the previous work of Alfin-Slater *et al.* (1957), Emken (1984) and Guesnet *et al.* (1986) in which EFA deficiency was shown to impair reproductive functions. Further physiological and biochemical investigations are needed on the role of hydrogenated fat in diets with marginal contents of EFA.

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Reply to letter by Neale & Norton

Our interpretation of results questioned in the letter of Drs Neale and Norton is based on comparisons of observations in animals given diets containing butterfat (BF) or hydrogenated vegetable oil (HO) in which linoleic acid contributed 0·38 and 0·54% of

dietary energy respectively. These calculations are based on a gross energy of 15 kJ (3·6 kcal)/g for the diet and 38 kJ (9 kcal)/g for linoleic acid.

The BF diet, which provided the lowest proportion of essential fatty acids of any of the experimental diets, did not give rise to signs of essential fatty acid (EFA) deficiency such as impaired growth, dermal lesions, decreased litter size or abnormal sperm morphology. Therefore, there is no practical reason to consider the HO diet, which provided more linoleic acid than the BF diet, as EFA deficient in itself. As judged by published results (e.g. Pudelkewicz *et al.* 1965; National Research Council, 1975) we should consider the linoleic acid content of the HO diet as 'marginal'. Therefore, by comparison with diet BF, the effects that we observed with the HO diet on reproductive performance of our experimental animals may be caused by other components of this fat, which we suggest are the high concentrations of isomeric fatty acids.

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