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NUTRITION BALANCE TECHNIQUES AND THEIR LIMITATIONS

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Techniques in energy metabolism studies and their limitations

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The determination of the calorific value of the material stored in or lost from the body during an interval of time can be made in two ways: by the comparative slaughter method and by the balance method. In the comparative slaughter method at the beginning and at the end of the time interval the heats of combustion of the entire bodies of two precisely similar animals are determined, and the retention of energy is determined by difference. Apart from identical twins, no two animals are precisely similar, so the comparative slaughter technique necessarily involves slaughter of fairly large groups of animals if the error attached to the estimate of retention is to be kept small. The method is suited to studies with small laboratory animals and poultry (Davidson, Mathieson, Williams & Boyne, 1964). It is an expensive method full of technical difficulty due to sampling problems when applied to large animals (Mitchell, Kammlade & Hamilton, 1928; Milford & Minson, 1965; Morris & Moir, 1963) and cannot be applied to man. The comparative slaughter method, however, does provide a direct measure of retention, limited only by analytical accuracy and by the basic assumption that initially the two groups of animals have the same energy content. With proper numbers and randomization of animals, this latter assumption does not lead to systematic error.

Balance methods to determine energy retention are of two types. In one the difference between the heat of combustion of the food and the sum of the heats of combustion of the excreta, including gases and the heat produced by the animal, is measured during an interval of time. This method is called the energy balance method. In the other, from the intake of carbon and nitrogen in food, all losses of these elements in the excreta and as gas are deducted to give estimates of the retention of C and N. Certain assumptions about the chemical forms in which C and N are stored in the body are then made to provide an estimate of the fat and protein and hence of the energy retained. When hydrogen retention is also determined by these same balance methods, estimates of fat, protein and carbohydrate retention can be achieved (Atwater & Benedict, 1904). These methods are called the C and N or C, N and H balance methods.

The advantages of balance methods are that they do not involve destruction of

the animal for they can be repeated time and again with the same animal, and theoretically they can measure small retentions of energy during short intervals of time. None of the balance methods is, however, free of basic assumptions which could give rise to systematic error. Furthermore, balance techniques in general are very prone to systematic error due to deficiencies in experimental techniques. In addition they are subject to errors of a statistical type, that is random errors largely arising from sampling difficulties.

Random errors

With ruminants which produce methane the random error attached to an estimate of energy retention by the energy balance method depends on the random errors attached to each of the five terms which contribute to it. These errors can be regarded as of two types, firstly analytical and instrumental errors and secondly errors which arise because the production of faeces and urine and part of the loss of methane by belching are discontinuous acts. Although the mean rate of production of faeces, urine and methane obtained from measurements made during long periods of time may be constant, short periods of observations can reveal deviations from these mean rates. With heat production too, day-to-day variation usually exceeds variation due to instrumental error. This day-to-day variation in heat production may be quite real, reflecting differences in the activity of the animal. In an assessment of the effect of a dietary regimen, however, all these day-to-day variations whatever their cause can be regarded as deviations from an overall constant rate characteristic of the regimen. These errors are called sequential errors.

Table 1 summarizes information accumulated in my laboratory about the analytical and sequential errors which occur in experiments to determine energy retention in sheep, and their resultant effects on the random error attached to an estimate of energy retention. Clearly, expressed as a percentage of the true retention, these errors are meaningless, for they approach infinity as retention approaches zero. Errors expressed as a percentage of the total intake are, as Schiemann (1958) has indicated, more meaningful. Table 1 shows that as a result of considerable care in analytical work the analytical and sampling error attached to the estimate of retention is quite small—about $\pm 1\%$ of the intake. In practice, however, this value is reached only when long periods are used, because of the considerable effect of sequential errors. It has been our practice to work with periods 4–5 days in length. This means that the random errors attached to retentions in sheep are about ± 40–50 kcal/24 h. Since the faecal error is one of the larger components, collection of faeces is often prolonged to 8 or 10 days, which further reduces the random error of the mean retention. As pointed out by Graham, Blaxter & Armstrong (1958) in agreement with Schiemann's (1958) analysis of calorimetric studies by Kellner & Köhler (1900), the C and N balance method, because it involves eight analytical measurements, leads to greater errors than does the energy balance method. We find errors to be about 30% greater for the C and N balance method than for the energy balance method.

Table 1. *The effects of random errors due to analytical causes and of random errors due to periodicity of excretion and other biological causes on the accuracy of estimates of energy retention made in energy balance experiments with sheep**

Component and reference	Nature of analytical and biological errors	Mean value and error due to analytical causes alone (kcal/24 h)	Error (kcal/24 h) due to combination of analytical causes and to biological causes when length of period in days was: 1	2	4	8
Intake (1)	Daily amounts of food (1000 g/day) are weighed at one time to ±0·5 g. The calorific value of the bulk is determined with an accuracy of ± 0·5% of the mean value	4091±21·6	±21·6	±21·6	±21·6	±21·6
Faecal excretion (1, 2)	Faeces (942 g/day) is weighed to ± 0·5 g and calorific value determined to ±0·5% of the mean value. The variation in the total weight of dry faeces collected is ±23 g and independent of the length of the period. That is errors of collection are compensating ones	1196±19·0	±92·1	±46·1	±23·8	±13·0
Urine excretion (3)	Urine (500 g/day) is weighed to ± 0·5 g and calorific value determined to ±2·2% of the mean value. Analysis of day-to-day variation shows the total error to be ±70 kcal/24 h and to be independent of length of period	204± 6·1	±70·0	±35·0	±17·5	± 8·7
Methane (4)	The instrumental and analytical error is ±2·2% of the mean value, and the day-to-day variation ±7·2%. This includes analytical error and is probably random	327± 7·8	±23·5	±16·5	±11·7	± 8·3
Heat production (1, 5)	Instrumental accuracy is ±1%. Analysis of serial experiments suggests the day-to-day error is ±52 kcal/24 h and has been taken to be random	1940±19·4	±52·0	±36·6	±26·0	±18·4
Retention	Errors are taken to be the square root of the sum of the separate variances. This assumes no correlation of errors	424±36·3	±131	±73·7	±46·4	±33·5
Error as a percentage of:						
Intake		± 0·9	± 3·2	± 1·8	± 1·1	± 0·8
Retention		± 8·5	±30·9	±17·4	±10·9	± 8·0

References: 1. Graham, Blaxter & Armstrong (1958); 2. Blaxter, Graham & Wainman (1956); 3. Blaxter, Clapperton & Martin (1966); 4. Blaxter & Clapperton (1965); 5. Blaxter & Graham (1955).

*The mean retention in this experiment of +424 kcal/24 h is about a third of the maximal retention that can be achieved when a sheep is fully fed. The percentage errors attached to the retention are clearly minimal at extreme values for retention and increase as the retention tends toward zero.

Obvious systematic errors

Given that analytical techniques are absolutely accurate and sequential errors are minimized by judicious choice of length of trial, systematic errors in energy

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balance and C and N balance trials largely arise from failures to collect the excreta completely or from allowing samples to deteriorate before analysis. The primary requisite in all balance work is absolute accuracy of collection, and this cannot be overemphasized. It has been our experience with the closed circuit chamber for cattle (Wainman & Blaxter, 1958) that 30–40 g of skin and hair debris together with some food dust is collected in the air filters every 4 days. Possibly 10 g of this is food dust, out of an intake of 20 kg a small error admittedly, but one which is systematic and is inevitably transferred to the estimate of body retention. Ensuring absolute accuracy in balance studies is difficult, and sometimes not easy to check. A rough check can be made in many trials by the device of weighing all the inputs into the calorimeter and all the outputs from the calorimeter during an interval of time. They must agree. An illustration of this checking method is given in Table 2 (Blaxter & Rook, 1956–7). The limitation of this method is the difficulty of weighing a large animal to within a few grams but the method will easily detect systematic errors which exceed the precision of the weighing machine used.

Table 2. *Gravimetric method of checking the results of a calorimetric trial (from an experiment with a calf by Blaxter & Rook, 1956–7)*

Input into the calorimeter in 4 days (g)		Output from the calorimeter in 4 days (g)	
Animal at beginning	36 450	Animal at end	35 846
Food	12 040	Faeces	272
Water	1 600	Urine	10 164
Oxygen	2 480	Water vapour	3 596
		Carbon dioxide	2 640
		Hair and skin debris	28
Total	52 570	Total	52 546
		Discrepancy: Value	24
		As a percentage of input	0·045

Systematic errors arising from assumptions

Direct calorimetry. With energy balance methods, determination of heat production by direct calorimetry might seem to be free of any bias. Calorimeters measure, however, not the heat production of the animal during a period but heat emission from the calorimeter and its contents during that period. Food which enters at a temperature different from that in the calorimeter, and faeces and urine which leave at a different temperature from that in the calorimeter, remove or add heat. The heat is proportional to the temperature differences, to the masses of materials and to their specific heats. Similarly if the body temperature of the animal or the temperature of its stall is different at the beginning from what it is at the end of the trial, heat actually produced is being stored or lost and so the heat emission is not equal to the heat production. Sometimes the increase in mass of the animal due to growth has to be taken into account (Cairnie, 1958; Cairnie & Pullar, 1959). Corrections can be made for these changes in heat content provided that temperatures can be measured accurately. The assumptions which may have to be made about specific heats cause negligible errors.

In some types of calorimeter in which heat loss by evaporation is determined from the mass of water vapour produced, an assumption has to be made about the heat lost when 1 g of water is vaporized. This is a multiple term and consists of, firstly, the latent heat of evaporation of the water, which varies with the temperature of the liquid water at lung and skin surfaces, secondly, the heat change required to expand the saturated water vapour as it cools from lung or skin temperature to air temperature and, thirdly, the heat required for isothermal expansion of the fully saturated vapour to the equilibrium saturation of the atmosphere in the calorimeter. The total heat required varies with conditions from 0·57 kcal/g to 0·66 kcal/g according to environmental conditions, the major determinant being the mean temperature at which the water is vaporized, a temperature not easily measured. The assumption that 'latent heat' is constant under all conditions could lead to systematic errors of up to 3% in the evaporative component of heat emission, or about 1% of the total heat in thermoneutral surroundings. The gradient layer instrument for direct heat measurement is free of this criticism (Benzinger & Kitzinger, 1949; Pullar, 1958).

Indirect calorimetry. In most experiments to determine retention heat production is not estimated from measurements of heat emission but from measurements of the respiratory exchange and urinary N excretion. The technique is sometimes called the 'RQ method'. Essentially the method consists of choosing two reference compounds containing C, H and O representative respectively of the normal O-rich and O-poor compounds which the animal oxidizes. If for example glucose and palmitic acid are chosen, from the equations for their oxidation one can write equations in terms of O_2 consumed, moles of CO_2 produced and kcal heat produced/mole of compound oxidized.

$$\text{Glucose:} \quad \triangle H = 673 = \alpha\ 6\ O_2 + \beta\ 6\ CO_2$$
$$\text{Palmitic acid:} \quad \triangle H = 2398 = \alpha\ 23\ O_2 + \beta\ 16\ CO_2$$

which on solution gives:

$$\triangle H \text{ (kcal)} = 86{\cdot}2\ O_2 \text{ (moles consumed)} + 25{\cdot}9\ CO_2 \text{ (moles produced).}$$

This can be expressed in terms of l. of CO_2:

$$\triangle H \text{ (kcal)} = 3{\cdot}85\ O_2 \text{ (l. consumed)} + 1{\cdot}16\ CO_2 \text{ (l. produced).}$$

This equation will estimate precisely the heat produced when any mixture of glucose and palmitic acid is oxidized. Choice of other reference compounds representing oxygen-poor and oxygen-rich compounds results in changes in the coefficients. The accuracy of this approach has been dealt with elsewhere (Blaxter, 1962). It suffices to state that when glucose and palmitic acid are reference substances the equation underestimates heat production by 5·0–6·4%; when polysaccharides are oxidized, it overestimates heat production; when aliphatic fatty acids with a chain length shorter than 16 C atoms are oxidized, it overestimates the heat of combustion of any dicarboxylic acids which are oxidized in the body by as much as 13% and it underestimates heat production by 4·4% when glycerol is oxidized. The reason for these divergencies is that this approach attempts to assess the heat of combustion of a complex organic molecule simply from the number and kind of its constituent atoms, ignoring completely the structure of the compound. The same difficulty occurs when compounds containing N are considered. Even so, the average

error incurred in using the RQ method is not great, particularly when reference compounds are chosen carefully to accord with the compounds oxidized. For example, with suckling animals, the reference carbohydrate is clearly lactose, the reference lipid a mixture of fatty acids resembling those in milk fat and the reference protein is casein. With ruminants the reference carbohydrate is cellulose rather than glucose, the reference fatty acids C_{18} acids and the reference protein some plant protein. Table 3 shows the extent of the discrepancy in estimated heat production incurred when a number of alternatives are used. Despite large differences in the individual coefficients used the resultant differences in calculated heat production are very small indeed.

Table 3. *Effect of differences in the magnitude of the factors used on the heat production calculated from the respiratory exchange*

		Factors			Heat production calculated when	
Author	Animal	O_2 (kcal/l. consumed)	CO_2 (kcal/l. produced)	N (kcal/g produced)	O_2=500 l. CO_2=500 l. N= 15 g	O_2=500 l. CO_2=400 l. N= 15 g
Weir (1949)	Man	3·941	1·106	−2·17	2490	2380
Gonzalez-Jimenez & Blaxter (1962)	Suckling calf	3·998	1·026	−1·60	2488	2385
Brouwer (1965)	Ruminant	3·866	1·200	−1·43	2511	2391
Maximal difference (%)		3·4	17·0	51·7	0·9	0·3

C and N balance. The factors used to convert retentions of C and N into retentions of energy are also based on the use of 'reference' compounds, in this instance body protein, usually muscle protein and body fat, usually the triglycerides of depot fat. Armbsy (1903) used muscle and depot fat as reference compounds and employed analyses made by Köhler (1900–1) to this end. Brouwer (1965) in a re-appraisal of these factors included more recent analyses of muscle by Franke & Weniger (1958). A different approach was made by Blaxter & Rook (1953). They abandoned the use of muscle protein and of purified depot fat as reference compounds and substituted values determined statistically by regressing the calorific value and C content of a variety of ash-free tissues of the body on their N content. The 'protein' base-line was then taken to be the mean C content and calorific value of the complex mixture of compounds which has a N content of 16·0%, and the 'fat' base-line the C content and calorific value of that mixture which contains no N. The approach is probably sounder than the approach by Köhler (1900–1), Armsby (1903) and Brouwer (1965) but, as shown in Table 4, the various factors proposed, despite their variation, do not make very much difference to the final estimate of energy retention. When N retention is very high, Blaxter & Rook's factors predict energy retention to be about 2% lower than that predicted by Armsby. Since muscle growth is associated with glycogen storage this seems reasonable but no direct proof is forthcoming.

Experimental evidence relating to the agreement of estimates

Direct versus indirect calorimetry. The agreement of the results of direct and

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Table 4. *The composition of reference body protein and reference body fat used by different authors to derive factors for the estimation of energy retention from C and N retention, the resulting prediction equations and the relative values of the estimates of energy retention derived from them*

Author	Composition of reference protein			Composition of reference fat		Equation for estimating energy retention*	Relative values of retention when the Armsby values are taken as 100, and when the percentage of the energy gained in the form of protein is:			
	N%	C%	kcal/g	C%	kcal/g		0	10	25	50
Armsby (1903)	16·67	52·54	5·70	76·51	9·50	H = 12·42 C − 4·96 N	100	100	100	100
Brouwer (1965)	16·00	52·00	5·70	76·70	9·50	H = 12·39 C − 4·64 N	99·7	99·8	100·0	100·35
Blaxter & Rook (1953)	16·00	51·20	5·32	74·84	9·37	H = 12·55 C − 6·90 N	101·0	100·5	99·5	97·9

*H in kcal, C and N in g.

indirect calorimetry was allegedly demonstrated by Rubner (1885) when he compared the results of direct calorimetry with dogs with those computed from C and N balance trials. In fact Rubner did not determine the C lost in faeces and urine but calculated it from N excretion. Agreement, however, was excellent, the mean discrepancy between observed and computed heat over 45 days of trial being −0·32% of the amount of heat measured directly. With ruminants, the results of the trials made with the Pennsylvania State College instrument, summarized by Armsby (1913) and by Kriss (1925), have been collected and the 129 observations are shown in Fig. 1. The comparison is between heat production determined by direct calorimetry and heat production estimated as the difference:

$$\text{Computed heat production} = \text{Heat of combustion of food} - \left\{ \text{Heat of combustion of faeces, urine and methane} + \text{Heat of combustion of fat and proteins stored as estimated from C and N balance} \right\}$$

The results apply to cattle which ate about 10 kg feed each day. Fig. 1 indicates that in 85% of the comparisons the differences between observation and expectation were less than ±400 kcal, which is approximately 1% of the intake of food energy. There is some evidence, from the report of Kriss (1925) on techniques, that N and C may well have been lost in the methods used to collect and sample the faeces and urine. Even so, the distribution of deviations in Fig. 1 does not suggest any gross error in the combined technique.

Energy retention from energy balances using indirect calorimetry and the C and N retention technique. Good agreement has been obtained in our work with sheep and cattle between estimates of retention obtained by C and N balance methods and energy balance methods with heat determined indirectly from O_2 consumption and the RQ. It used to be stated (see Møllgaard, 1929) that such agreement was evidence of absolute accuracy; indeed the comparison was usually stated to measure 'apparent

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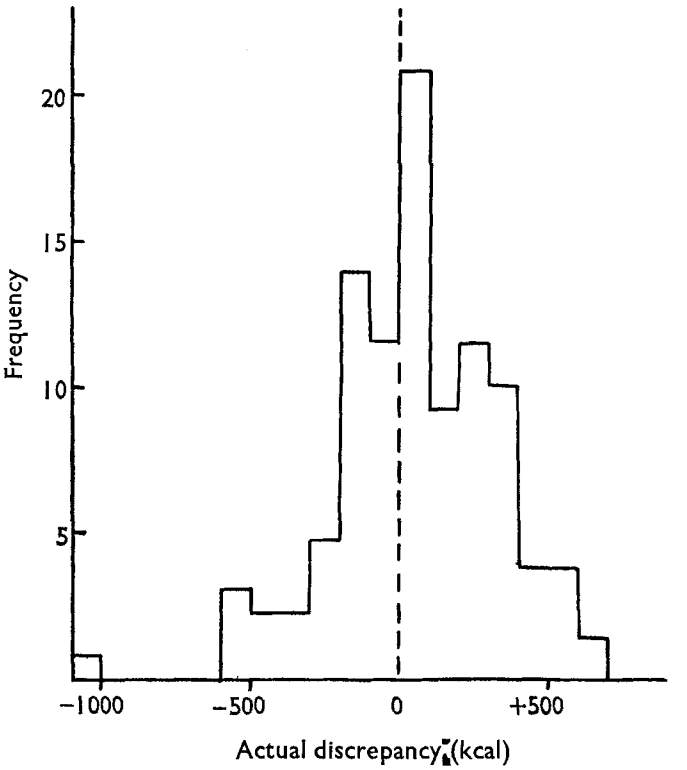

Fig. 1. The differences between computed and observed heat production in 129 experiments with ruminants carried out in the direct calorimeter at Pennsylvania State College.

deviation from the first law of thermodynamics'. As rightly pointed out by Hoffmann (1958), agreement is not proof of absolute accuracy. The same weights of food, faeces, urine and methane are used to arrive at estimates of energy, C and N intake and losses, and the CO_2 production of the animal enters into both the calculation of heat and the determination of C balance. Agreement between indirect measurements merely indicates that analytical precision was of a high order, that errors in feeding and collection of excreta did not disturb the overall ratio of energy to g C too greatly, and that errors of assumption were probably common. Agreement of this sort is less satisfactory than agreement between direct heat measurements and the estimates of heat made by indirect calorimetry.

Clearly, to inculcate faith more direct proof is needed that the balance methods give correct estimates of energy retention within the $\pm 1\%$ limits of error already discussed. The most acceptable proof is demonstration of agreement of the results of balance trials with the results of properly conducted and adequately replicated comparative slaughter trials in which the same animals as those used for the balance trials are killed to form the second group.

Comparative slaughter and calorimetry. No detailed reports of experiments have been traced in which the unequivocal evidence obtained by comparative slaughter trials has been compared with that from calorimetric trials. In one such trial at the

Rowett Institute (Rowett Research Institute, 1965), however, direct measurement of heat loss of chickens in a gradient layer calorimeter agreed to within ±5% of the heat loss computed as the difference between food intake, and excreta loss plus energy retained from body analysis.

Comparison of calorimetric estimates of retention with weight gains. Another method of checking, slightly less rigorous than the direct comparison, is to compare long-term cumulative estimates of energy retention, in which the energy can be divided into fat and protein, with body-weight changes during the same period. With cattle Schiemann, Hoffmann & Jentsch (1961) have made such comparisons, and recently Clapperton and I (unpublished) have made such comparisons with sheep. The results of the German experiments in which maintenance rations were given for 12 months are summarized in Table 5. Considerable error attaches to the estimation

Table 5. *Results of experiments by Schiemann* et al. (1961) *in which four oxen were given constant diets for* 12 *months and cumulative fat and protein deposition was determined by C and N balance methods*

		Calculated deposition from C and N retention		
Ox no.	Initial weight (kg)	Dry protein (kg)	Dry fat (kg)	Observed body-weight gain (kg)
05	752	13·4	54·0	28
07	709	13·6	37·0	17
012	641	21·2	29·6	46
013	517	21·8	44·1	67

of weight gain in ruminants because of the large amounts of water they consume and of faeces and urine they produce at any one time. It must be assumed, however, that precautions were taken to minimize these changes. The animals were old and very large. Their true body gains undoubtedly contained very little protein, and probably little error is caused by assuming that the gains had a calorific value of 9·0 kcal/g. The amounts of protein and fat computed to have been stored in three instances out of four exceeded the gain. Making the approximation that the gains were largely of fat, that is that the N retention figures are the more likely to be subject to systematic error, the discrepancy between energy gains computed from C and N balances and those estimated from weight gains were +317, +262, +34 and −91 Mcal/year. The intake of energy per year was about 10^4 Mcal. The errors expressed as a percentage of intake were thus +3·2, +2·7, +0·3 and −0·9. The statistical errors of the retentions of C given by Schiemann *et al.* (1961) ranged from ±1·0 to ±1·4% of the intake.

Table 6 and Fig. 2 summarize the results of experiments with eight sheep made over 10 months. In these experiments the amount of diet given was constant throughout, and, in addition to measurements of energy retention, N retention and weight gain, the growth of wool and the fibre and wax content of the wool were determined throughout. Computed gain, which consists of the sum of wool growth, body fat

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Table 6. *Results of eight energy balance (EB) trials in which sheep were given constant amounts of food for* 10 *months (Blaxter & Clapperton,* 1966, *unpublished)*

Sheep	Initial weight (kg)	Energy retained (kcal/day)	Weight gain items (g/day)				Observed weight gain (g/day)	Gain or loss in 10 months (kg)	
			Wool growth	Body fat	Body flesh	Total		From EB	Observed
R1	44·1	− 73·4	2·9	− 9·3	− 5·2	− 11·6	− 23·5	− 3·2	− 6·6
R2	45·8	− 48·8	2·5	− 5·9	−30·1	− 33·5	− 33·4	− 9·4	− 9·4
W1	53·6	− 55·2	5·0	− 8·3	−19·2	− 22·5	− 11·6	− 6·3	− 3·2
W2	43·1	+166·7	4·2	+15·9	+ 3·2	+ 23·3	+ 29·4	+ 6·5	+ 8·2
B1	48·9	+429·7	5·6	+42·9	+28·8	+ 77·3	+ 66·9	+21·6	+18·7
B2	45·0	+447·8	6·7	+44·3	+39·6	+ 90·6	+ 59·6	+25·3	+16·7
Y1	48·9	+667·2	7·1	+67·4	+31·8	+106·3	+ 91·2	+29·8	+25·5
Y2	50·8	+622·9	7·5	+62·6	+40·0	+110·1	+114·6	+30·8	+32·1

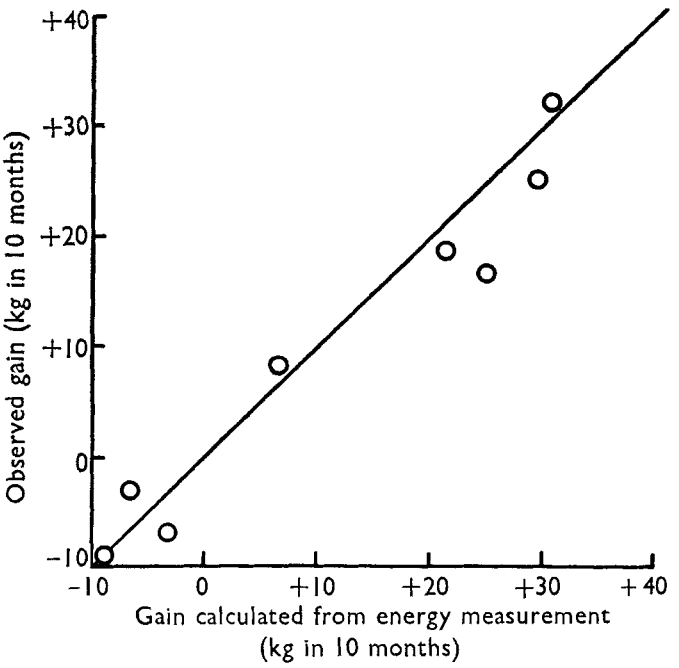

Fig. 2. The agreement between estimates of the retention of protein by sheep, measured by the carbon–nitrogen balance methods and by energy balance methods in which the heat was determined indirectly from oxygen consumption.

deposition and body protein deposition, on the assumption that each g N stored represents 31 g body-weight, has been compared with determined body gain. Statistical analysis of these results showed that the relation between the two estimates of weight did not differ from direct proportionality nor was there any significant bias. The standard deviation of differences was ±15·2 g/day or 4·2 kg in 10

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months. The error of estimating by weighing the true body-weight change uncomplicated by changes in gut contents, bladder contents or the moisture content of the fleece is probably ± 500 g. The calorific value of gains was probably close to 5·0 kcal/g. If the error attached to the retention of energy was ± 50 kcal/day (see Table 1) the standard deviation of the difference between observed and calculated gain can be expected to be $\pm 2{\cdot}9$ kg: if the error of the retention is ± 75 kcal/day the expected standard deviation of differences is $\pm 4{\cdot}3$ kg. It would appear that the divergence between observed gains and those computed from balances is largely that to be expected from the errors attached to the estimate of energy retention.

In conclusion, it appears that provided great care is taken in the very considerable work involved in balance trials there is no reason to suppose that energy retention measured by these methods is inaccurate or biased in any significant way. Their limitation lies solely in the prodigious amount of work they entail.

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